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# GIBBERELLINS REGULATE CELL DIVISION AND PARTHENOCARPIC FRUIT SET IN CITRUS

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**GRAZIE A TUTTI**

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# ABBREVIATIONS

**FAO.** Food and agricultural organizaton

**GAs.** Gibberellins

**GA.** Gibberellin

**IAA.** Indolacetic acid

**ABA.** Abscisic acid

**OH.** Hydroxyl

**GGPP.** Geranylgeranyl diphosphate

**IPP.** Isopentenyl diphosphate

**MVA.** Mevalonic acid

**CPS.** *ent*-copalyl diphosphate synthase

**CPP.** *ent*-copalyl diphosphate

**KS.** *ent*-kaurene synthase

**KO.** *ent*-kaurene oxidase

**KAO.** *ent*-kaurenoic acid oxidase

**GA20ox.** Gibberellin 20-oxidase

**GA3ox.** Gibberellin 3-oxidase

**GA2ox.** Gibberellin 2-oxidase

**CycA1,1.** Cyclin family A.

**DAA.** Days after anthesis; **- DAA**=Days before anthesis

**DAP.** Days after pollination

**BBCH.** Citrus Biologische Bundesanstalt, Bundessortenamt and Chemical Industry

**UPLC-MS/MS.** Ultra high pressure liquid cromatography, mass spectrometry

**RT-PCR.** Real time-polymerase chain reaction

**$GA_3$.** Gibberellic acid

**PBZ.** Paclobutrazol

# ABSTRACT

Fruit set and growth is regulated by hormones interaction, gibberellins (GA) being a main part of the process in *Citrus*. Actually, it is well known the time-course of GA content in the citrus ovary during flowering and its correlation with fruit set in both seeded and seedless species. However, GA endogenous role is uncertain. Is GA in the ovary a direct regulator or an indirect part of the hormonal stimuli that reactivates and maintains cell division triggering fruit set? While in seeded varieties GA synthesis that occurs in fertilized ovules is the primary stimulus controlling early ovary development, is there any specific tissue-dependent regulation of parthenocarpy? While initiation of GA synthesis is thought to be autonomous in the sterile parthenocarpic species, it is unclear whether it is autonomous or stimulated in the fertile self-incompatible species, which present a wide variation in their parthenocarpic ability.

Attending to the aforementioned, in this PhD Thesis, the following hypothesis was tested:

The autonomous synthesis of GA in the pericarp directly activates and maintains cell division in the ovary, promoting the flower to fruit transition in parthenocarpic *Citrus*.

The main findings are:

1. In the parthenocarpic cultivars, GA biosynthesis is first located in the whole pericarp and the ovules during the flower to fruit transition; later, during the fruit cell division developmental stage, GA biosynthesis is located in the endocarp and growing juice sacs.

2. The autonomous activation of GA biosynthesis directly stimulates *CYCA1,1* gene expression and cell division in the ovary walls (exocarp and mainly mesocarp) and in the endocarp (growing juice sacs), increasing ovary growth rates and leading to higher parthenocarpic fruit set.

3. Fertile self-incompatible cultivars (i.e. Clementine mandarins) present pollination-independent facultative parthenocarpy, rather than stimulative parthenocarpy, with its ability to set being dependent on endogenous $GA_1$ levels in the ovary at anthesis, which is triggered autonomously depending on the genotype.

# INTRODUCTION

# 1. CITRUS

## *1.1. Taxonomy, origin and expansion*

In the first classification of plants (1737), Linnaeus arranged the genus *Citrus* into three species: *C. medica* (cedar and lemon), *C. aurantium* (sour and sweet oranges and grapefruit) and *C. trifoliata* (Poncirus). Thereafter, Linnaeus separated grapefruit from *C. aurantium* and in collaboration with Osbeck three species were proposed, *C. grandis* (grapefruit), *C. sinensis* (orange) and *C. limonia* (Rangpur lime). Later, Burmann proposed lemon as an autonomous species (*C. limon*) (Calabrese, 2004).

From this moment and throughout the years, it has been appearing several approaches of Aurantioideas classification, to which citrus belong. The Aurantioideas subfamily belongs to the Embriophyta Siphonogama division, Angiospermae subdivision, Dicotyledonae class, Rosidae subclass, Rutanae superorder, and Rutales order, all encompassed in the family Rutaceae.

But it was not until the end of the 19$^{th}$ century when Hooker and Engler presented the *Citrus* taxonomic system based on the morphological characteristics and the supposed origin of these species. These authors proposed the existence of 13 genus and 11 species. In the mid 20$^{th}$ century, Swingle improved the taxonomic system including two families, Clauseneae and Citreae, based on several morphological characteristics. In his later work, Swingle (1967) reviewed the taxonomic system suggesting a division of the Citreae families into three sub-families, one of which, the Citriniae, contains the cultivated citrus: ***Fortunella, Poncirus*** *and* ***Citrus***.

The species of the genus *Citrus* are the most important from an agricultural point of view. The most common species are ***C. aurantifolia*** (Christm.) Swing. Mexican lime, ***C. latifolia L.*** Lime Tahiti, ***C. aurantium L.*** Sour orange, ***C. grandis*** (L.) Osb. Pummelo, ***C. limon*** (L.) Burm. Lemon, ***C. paradisi Macf.*** Grapefruit, ***C. reticulata Blanco.*** Tangerine, ***C. sinensis*** (L.) Osb. Sweet orange. Citrus fruits are produced for fresh consumption and for processing into juice.

However this is not the only botanical classification of citrus currently used. According Tanaka (1977), the anatomical differences among citrus merit a broader classification. This author included 162 species in the genus *Citrus*. Recent studies based on chemotaxonomy, morphology and genetics (Wu *et al*., 2014) suggest the existence of three affinity groups: *Citrus* medica, including *C. medica, C. aurantifolia* and *C. limon*; *Citrus reticulata*, including *C. reticulata*, *C. sinensis*, *C. paradisi*, *C. aurantium* and *C. jambhiri*; and finally, *Citrus maxima*, which includes only this single species. A fourth group, the *Citrus halimii,* has no agronomic interest. Currently, the botanical classification used is not uniform, and while Swingle's criterion is the most common, Tanaka's criterion has been fully accepted for several species. Such is the case of the species *C. unshiu* Marc., Satsuma mandarin, *C. latifolia* Tan., acid lime, *C. clementina* Hort. ex Tanaka, Clementine mandarin, and *C. reshni* Hort. ex Tanaka, or Cleopatra mandarin.

Citrus species originated in eastern Asia, in an area that extends from the Himalayan slopes to southern China, Indochina, Thailand, Malaysia and Indonesia, where the climate is humid, warm and subtropical. Cultivated citrus are selections from, or hybrids of, wild progenitor species whose identities and contributions to citrus domestication remain controversial. According to genomic studies (Wu *et al.,* 2014), citrus cultivated types derive from two progenitor species, *Citrus maxima* and *Citrus reticulata*. Although cultivated pummelos represent selections only from *Citrus maxima*, cultivated mandarins are introgressions of *C. maxima* into the ancestral mandarin species *Citrus reticulata*. Sweet orange is the descendant of previously admixed individuals, but sour orange is an F1 hybrid of pure *C. maxima* and *C. reticulata* parents, thus implying that wild mandarins were part of the early breeding germplasm.

To understand how citrus have been preserved to this day, faithfully respecting the essential characteristics of the species, it is interesting to note that these plants present apomixis, the ability to form nucellar embryos. However, the diversification process that has led to the species we know today was produced through two pathways: 1) genetic somatic mutation, and 2) hybridization (Calabrese, 2004).

The complexity of both the origin of citrus, which is probably not unique, and its expansion, is not surprising. Thus, the presence of citrus in ancient Egypt seems

obvious but has not been tested. It is possible that Greece facilitated the expansion of citrus fruit to Europe in the early Christian era, but this hypothesis is controversial since several authors think that it was a misinterpretation of Theophrastus (Agustí, 2003). It seems clear that different cultures had contact with citrus fruit from early on (60-70 C.E.), and all cultures, from the Romans, Greeks, Arabs and Asians contributed to its expansion in the Mediterranean region. The Spanish conquerors introduced citrus cultivation in America from as early as the 16$^{th}$ century. Calabrese (2004) and Agustí *et al.* (2014) have been summarized the origin and expansion of citrus.

### *1.2. Geographic distribution and economic importance*

Citrus are grown in most of the tropical and subtropical regions of the world between the latitudes 40°N to 40°S. However, large-scale, commercial plantations of citrus have developed almost exclusively in subtropical regions where temperatures are moderated by sea winds. These occur in two fringes around the world that extend roughly between 20°N and 40°N and S of the equator.

Citrus is the most economically important fruit worldwide, accounting for a quarter of all fruit production, totalling of 115 million tonnes in 2013 (FAO 2013). The leading producer is China with more than 22.9 million tonnes per year, followed by Brazil with 22.7 million tonnes and the United States with 10.4 million tonnes. These countries are the top three producers in the world, and devote most of their production to the processing industry. Processed citrus is headed by Brazil, which produces 14.8 million tonnes per year, followed by the United States, with 6.9 million tonnes, and in third place is Argentina that processes 1.4 million tonnes (more than 75% corresponds to lemon) (FAO 2013). Spain ranks as the sixth largest producing country at 6.6 million tonnes, and it is the main producer for fresh consumption, (3.6 million tonnes a year). Italy produces about 3.2 million tonnes and dedicates most of this to domestic consumption (FAO 2013).

With a production totalling 70.6 million tonnes, orange is the most widely grown species in the world, accounting for 65% of the volume of citrus, followed by tangerine (25 million tonnes), lemon (12.8 million tonnes) and grapefruit (6.3 million tonnes) (FAO 2013).

In the Mediterranean basin, citrus production is mainly for fresh consumption in European Union, where seedless fruits are preferred. Thus, oranges and mandarins grown in this area are completely seedless, if grown in isolation, because of genetic sterility or a self-incompatible system; however, when some of these parthenocarpic *Citrus* cultivars are fertilized through cross-pollination, fruit and seed yield increases, and seed presence critically reduces fruit quality. Therefore, bud mutations are selected according to their parthenocarpic ability. This is the case of mandarins, particularly of Clementine mandarins, which are highly valued by consumers because the main cultivars set seedless fruits. Nevertheless, since it matures from late October to late January, in the northern hemisphere, nowadays a number of late maturing (later than February) hybrid mandarins, such as 'Fortune', 'Ortanique' or 'Afourer', are grown to extend the mandarin commercial period. Most of these hybrid mandarins can cross-pollinate with Clementine mandarin cultivars and other *Citrus* species and, consequently, all of them set seedy fruits, an undesirable characteristic that greatly reduces grower returns.

### *1.3. Main species*

The agronomic classification of the genus *Citrus* is composed of five varietal groups: (I) Orange, (II) Tangerines (which includes all species of mandarins and their hybrids), (III) Lemons and Limes, (IV) Grapefruit and (V) other citrus fruits (FAO, 2013). Agustí (2003), Calabrese (2004), and Agustí *et al.* (2014) provide a detailed revision of the main characteristics of cultivated citrus species.

**Sweet Orange (*Citrus sinensis L. Osb)***

Sweet orange is the most widely cultivated species of the genus *Citrus* and may be separated into three groups: 1) common oranges, 2) navel oranges and 3) pigmented oranges.

Group 1: Common oranges

The common orange is the main orange variety worldwide from the economic point of view, and it is mainly produced for juice production. The group of common oranges branches into three subgroups according to the harvesting period: early white as 'Salustiana' and 'Hamlin', the mid-season as 'Pineapple' and white late as 'Valencia' and 'Pera'. The most notable characteristics of this group are its late ripening and organoleptic quality with lower acidity compared to other groups, and a natural tendency to alternate bearing. For juice production, 'Pera' is the variety most widely cultivated in Brazil, the world's leading producer of juice.

Group 2: Navel oranges

Navel oranges are the second most widely planted group and are mainly marketed for fresh consumption. This group is characterized by having a small secondary fruit embedded in the stylar end of the primary fruit. This aspect gives the fruit his name. Navel oranges are the earliest maturing of orange varieties, producing seedless fruit of large size, spherical in shape, with deep orange colour, and a rich, sweet and pleasant flavour. Navel group cultivars represent a significant proportion of the citrus production of Australia, Argentina, California, Morocco, Spain, South Africa, Turkey and Uruguay. The varieties of this group have male and female gametic sterility and, therefore, its fruits are parthenocarpic and have no seeds. Due to its high organoleptic quality, production is mainly destined for fresh consumption. The first navel originated in Brazil by mutation of the variety 'Selecta', belonging to the common group. All existing cultivars in the navel group have originated by mutation. The 'Washington navel' cultivar is the most widely grown. Other important varieties of this group are 'Navelina', 'Newhall', 'Lanelate' and 'Navelate'.

Group 3: Pigmented oranges

Pigmented oranges or blood, constitute a non-economically important group and plantings are limited to areas with Mediterranean-type climates, mainly in Italy and Tunisia. This group of varieties develops deep red flesh due to the anthocyanin

pigments, which can also develop in the rind. These pigments are related with hot days and, above all cold nights. The main cultivars include early maturing cultivars 'Tarocco' and 'Gallo', medium maturing 'Tarocco Ippolito' and 'Sciara', and late maturing cultivars 'Sanguinello' and 'Moro', the latter being the primary variety for juice processing.

**Tangerines**

In some botanical classifications, the generic name 'mandarin' includes a group of citrus belonging to different species, while Swingle's classification (1967) includes all in a single species, *Citrus reticulata* Blanco. Mandarin taxonomy is very complex because numerous biotypes and hybrids exist. Swingle distinguishes 5 groups, while Tanaka (1977) recognizes up to 36. The most economically important mandarins are Satsuma (*C. unshiu* Marc.), Clementine (*C. clementina* Hort. ex Tan.), 'Ponkan' (*C. reticulata* Blanco), 'Dancy' (*C. tangerine* Hort. ex Tan.), and hybrid-like common mandarins such as 'Nova' [*C. x clementina* (*C. paradisi x C. tangerine*)], 'Ortanique' (*C. reticulata x C. sinensis*) and 'Afourer' (Murcott x Clementine). The main producers are China, Spain, Brazil and Japan (FAO, 2013). Satsumas predominate in Japan while clementines are characteristic of the Mediterranean area.

Satsuma mandarins are mainly grown for fresh consumption, but are suited to processing for juice and for canning segments in syrup or juice. The Satsuma tree is vigorous and very productive, and exhibits a spreading habit with long dropping branches. The leaf is large, slightly leathery, dark full green, and has a prominent midvein. Fruit is moderately large compared to other mandarins, slightly flattened in shape, smooth peel, yellowish, of acceptable flavour, and seedless. Size, shape, colour and flavour mainly depend on the growing area, fruit produced under cooler conditions being usually small, flattened and of deep orange peel colour. Satsuma cultivars are commonly divided into two groups based on their maturation date. The earlier maturing varieties include 'Miyagawa' wase, which is the most widely grown Satsuma in Japan,'Okitsu' wase, which originated as a nucellar seedling by controlled pollination from 'Miyagawa' fruit, widely grown in Japan and Spain, and 'Clausellina',

an ‘Owari’ bud mutation originating in Almassora, Castellon, Spain. These mandarins frequently tend to develop parthenocarpic fruits.

The origin of Clementine mandarin is uncertain, but it is believed to have originated in China and selected in Algeria. In Spain and Morocco ‘Clementine’ mandarin has become the fastest expanding citrus variety over past five decades. ‘Clementine’ mandarin is also grown in Argentina, Uruguay, California, South Africa and Peru. The tree is densely foliated, small to large size depending on the cultivar and has regular high yields. Leaves are lanceolate. Fruit is medium to small in size, easy to peel, with excellent flavour, and seedless, although cross-pollination with common oranges, grapefruit and hybrids, can lead to fruit with several seeds per tree. The ‘Clementine’ mandarin fruit is sensitive to rainfall and relative humidity, developing a very fast senescence period that reduces external fruit quality. Several cultivars of ‘Clementine’ have been obtained by spontaneous bud mutation in Spain differing in time of maturation, tree size, fruit size and yield. One of the better mutations derived from Clementine is ‘Fina’. Fruit of ‘Fina’ is of excellent quality, deep orange-reddish and has very good organoleptic characteristics: pleasant aroma, tender, sweet and high juice content; however, it is very small. The rind is smooth and easy to peel. The fruit must be collected no later than mid to the end of December. ‘Clemenules’ mandarin is, nowadays, the most widely cultivated Clementine mandarin in Spain and Morocco. Similar to ‘Fina’, it has larger fruit, and it is somewhat more resistance to environmental conditions. Of the other mutations,‘Oroval’, ‘Oronules’ and ‘Marisol’ mature two to four weeks earlier, and ‘Hernandina’ one month later. Clementine mandarins produce, in general, weakly parthenocarpic fruit, requiring gibberellic acid ($GA_3$) sprays to achieve adequate fruit set and yields. However, ‘Marisol’ generally tends to develop parthenocarpic fruits.

**Lemons (*Citrus limon* L) and Limes (*Citrus latifolia*)**

The Mediterranean lemon we know today is very likely to be a hybrid of citron. Lemon cultivars sensitive are grouped in three types: Sicilian, Verna, and Femminello. Lemon trees are very to low temperatures and to fungal and algal diseases, thus it is not well suited for to humid subtropical or tropical regions. However, they do grow

well under the Mediterranean climate. Lemon trees tend to grow, flower, and produce fruit continuously throughout the year, but in Mediterranean climates trees have two major flowering periods, in spring and summer. Depending on distinct factors, the fruit is harvested either in autumn, winter or summer. In the Mediterranean area, the cultivated lemon varieties are mainly 'Eureka', 'Verna' and 'Femminello'.

Lime trees probably originated in the tropical Malay Archipelago. They are the most freeze-sensitive of all commercial citrus species and, thus, its cultivation is limited to the tropics and warm, humid subtropical regions of the world. There are two groups of limes: 'Tahiti' and 'Mexican' limes. The former includes the 'Persian' and 'Bears' limes, the latter includes the 'West Indian' and 'Key' limes.

**Grapefruit (*Citrus paradisi*)**

The origin of grapefruit (pomelo, toronja) is uncertain, but there is evidence that seeds were introduced in Barbados by the early settlers and in Florida (USA) at the beginning of nineteenth century from Cuba, Jamaica or The Bahamas. Grapefruit distribution is more limited than sweet oranges and mandarins. Grapefruit contains two groups, Common and pigmented cultivars. In both groups there are varieties with seeds, such as 'Duncan' (common grapefruit) and 'Foster' (pigmented grapefruit) and seedless varieties such as 'Marsh' (common grapefruit) and 'Thompson', 'Redblush' and 'Star Ruby' (pigmented grapefruit). The United States, China and South Africa are the top three producers in grapefruit (FAO, 2013). In Europe they have little economic importance.

## 2. FRUIT SET

Fruit set is defined as the transition of the quiescent ovary of the flower to a developing fruit. The onset of fruit development involves two processes, namely 1) fertilization of the ovule and 2) growth of the pericarp tissues and/or other structures of the pistil surrounding the developing seed. The process is based on the reactivation of cell division in the ovary after anthesis, thus requiring a considerable amount of energy that is satisfied from the import of photoassimilates into the fruit. Thus, photoassimilate limitation is a primary driver of flower and fruit abortion. Hormones play a major role in synchronizing fertilization and fruit growth. In seeded varieties, i.e. 'Pineapple' sweet orange, hormone synthesis which occurs in fertilized ovules is the primary stimulus controlling early fruit development (Ben-Cheikh *et al.*, 1997); in these varieties emasculation of the flower prevents seed formation and hormone synthesis, arresting fruit development and triggering abscission. However, the exogenous application of growth regulators to emasculated flowers can restore fruit development and, more importantly, the existence of parthenocarpic fruits indicates that ovule fertilization is not an absolute requirement for fruit set. Thus, in parthenocarpic *Citrus*, ovule fertilization and ovary development, which are hormonally regulated, are uncoupled (Distefano *et al.*, 2011). Consequently, anatomical changes in the ovary take place in the same way and at the same time in un-pollinated or cross-pollinated flowers. Ovary abscission occurs afterwards, the intensity of this depending on the parthenocarpic ability of the cultivar (Talón *et al*., 1992). This suggests that ovary growth at the onset of the cell division stage is not dependent on pollination but rather it is developmentally controlled (Distefano *et al*., 2011). Monselise (1977) suggested that hormone synthesis in parthenocarpic fruits might occur in ovary walls, replacing that of the seed and stimulating parthenocarpic ovary development. However, this hypothesis remains unproved and the regulatory role of pericarp tissues in fruit set is unknown.

In citrus, abscission of reproductive organs is almost continuous from the blooming period to the cell division stage of fruit development. However, three abscission stages of flowers and fruits can be distinguished (Agustí *et al.*, 1982): the first abscission stage occurs during flower bud formation at preanthesis; the second

stage induces flower and ovary abscission in the days following anthesis and it also determines fruit set; finally, the third wave of abscission affects developing fruitlets during the physiological fruit drop which determines the final yield. The intensity and duration of each stage depend on the competition between developing organs, determined by endogenous factors, mainly nutritional and hormonal. This competition is established from the beginning of flower development. Comparing trees with different flowering intensity, Agustí *et al.* (1982) showed that 1) for low flowering intensity trees, a high percentage of flowers set a fruit and the major abscission occurs during the physiological fruit drop; 2) as flowering intensity increases, abscission anticipates to the flower stage and the percentage of fruit set significantly decreases. However, it is not only the number of flowers that will determine fruit set, but also the ability of each fruitlet to remain on the plant, which depends on its sink strength. Fruit set is inversely related to fruitlet growth rate. Growth rates are higher for fruits growing on shoots with leaves than those fruits from leafless shoots. Four to five weeks after anthesis, leaves become a source of carbohydrates and plant growth regulators, the shoot becoming self to develop the fruit (Spiegel Roy and Goldschmidt, 1996). In this sense, the presence of leaves plays a major role synthesizing and exporting metabolites to the developing fruit.

### *2.1. Anatomy of the flower and fruit*

Citrus flowers grow single or in clusters and have 5 sepals, 5 petals, 20–40 stamens and a single ovary with 10-12 fused carpels (segments) containing 4–8 ovules each. The style is long and has one stigma (Fig.1A). The biological function of a flower involves hosting the process of sexual reproduction that plants develop to perpetuate their species. During pollination, the pollen grain is carried to the stigma, where it germinates, the pollen tube growing down the style through the stylar canals and entering the ovary where it fertilizes the ovule. After fertilization, the ovary containing the newly fertilized ovules becomes a fruit (Fig. 1B-C), which is responsible for protecting the seed and facilitating its dispersal to produce a new plant. Numerous internal and external factors influence the fruiting process, some of which are still poorly understood.

The fruit is a type of berry called hesperidium and varies in shape and size depending on the species and varieties. The fruit peel contains numerous oil glands and two well-defined tissues. The external part of the peel is the *exocarp*, called *flavedo*, and the internal part of the peel is the *mesocarp* and is called *albedo*. The flavedo is composed of the cuticle-covered epidermis and the compactly arranged parenchyma cells adjacent to it (Schneider, 1968). At fruit maturity, it varies in colour from orange and reddish orange (oranges) to deep orange (mandarins) or green-yellow (lemons, limes and grapefruit). The albedo is a white and spongy tissue that separates the flavedo from the segments. The *endocarp* is the inner side of the pericarp and a portion of the locular membrane. It is composed of the inner epidermis and several layers of compactly arranged parenchyma cells adjacent to it. Juice vesicles can be differentiated from the endocarp epidermis; about the time the petals open until after the style falls, by oblique, anticlinal and periclinal divisions, and compactly fill the locules at fruit maturity (Schneider, 1968). Seeds are ovate to roundish in shape, mono- or poly-embryonic, with the colours of cotyledons ranging from white (oranges and grapefruit) to green (mandarins) (Schneider, 1968).

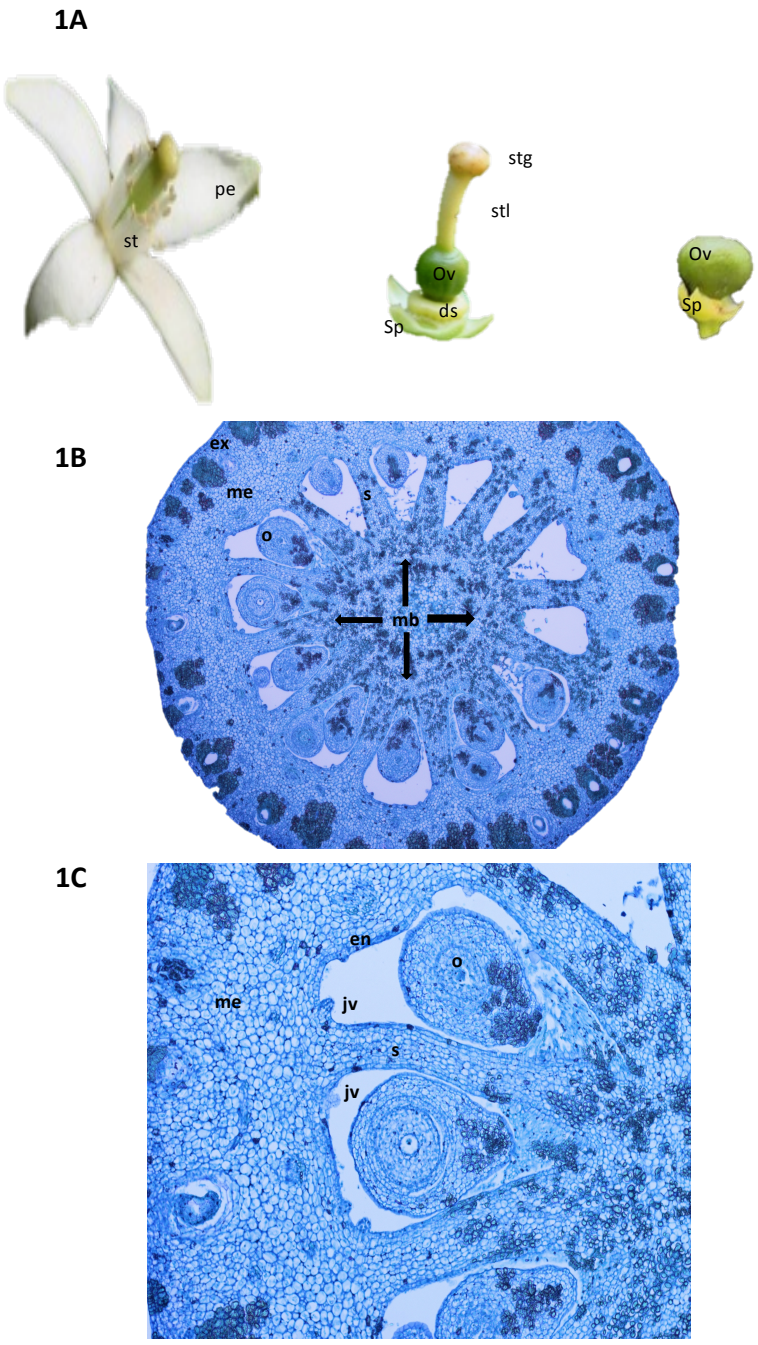



**Figure 1**. (A) Anatomy of the flower to fruit transition; (B, C) histology of the ovary. pe: petals; st: stamens; stg: stigma; stl: style; ov: ovary; ds: disc; sp: sepals; ex: exocarp; me: mesocarp; en: endocarp; jv: juice vesicles; o: ovules; s: septa; mb: marginal bundles.

## *2.2. Pollination and parthenocarpy*

Pollination is the transfer of pollen from the anthers to the stigma, where the pollen grain germinates and the pollen tube grows transporting the sperm nucleus to the ovary. Fecundation (or fertilization) consists of the fusion of a sperm nucleus with an egg nucleus. When a pistil is pollinated by pollen from the same plant or another genetically identical plant it is called self-pollination. If further fertilization occurs, it is called self-fertilization. Cross-pollination takes place when pollen comes from a genetically different plant and if fertilization occurs, it is also called cross-fertilization (Schneider, 1968).

Several agents are able of transporting pollen between flowers but pollination type depends to a large extent on the physical characteristics of the pollen. In the case of *Citrus*, pollen is heavy, viscous and sticky, characteristic of pollination by insects or entomophilous. Honeybees (*Apis mellifera*) are the main pollinator of these species, representing more than 90% of pollinating vectors (Krezdorn, 1970; Pons *et al.*, 1996).

Parthenocarpy occurs when an ovary develops without fertilization of their ovules and therefore without seeds. This phenomenon is common in many citrus species and varieties.

### *2.2.1. Origin and types of parthenocarpy. Sterility*

There are two types of parthenocarpy: stimulated and autonomous. In stimulated parthenocarpy an external stimulus is required for the development of the ovary after anthesis. Sometimes this role is awarded to pollen grain germination and pollen tube development, but in any case reaching fertilization. This is the case of self-incompatible genotypes of several species presenting homogenetic sterility, such as Apple (*Malus pumila*) (Saito *et al*., 2007), barberry (*Berberis vulgaris L*. cv. Asperma) (Ebadi *et al*., 2010) and grape (*Vitis Vinifera L*.) (Bonanda, 2011), and also for tomato (*Solanum lycopersicum*) transgenic lines (Schijlen *et al*., 2007). At the moment, it is unknown whether this type of parthenocarpy naturally occurs in some species of the genus *Citrus*, although the use of sterile-irradiated pollen is thought to stimulate parthenocarpy (Vardi *et al.,* 1988).

On the other hand, the development of the ovary without any external stimulus is known as autonomous parthenocarpy. Environmental factors can induce parthenocarpic fruit set, directly or indirectly affecting pollination, stigma receptivity, pollen tube development or ovule development. However, the main reason for this alternative to fertilization is genetic sterility, being gametic, cytological or homogenetic. Gametic sterility is the inability to produce fertile ovules or pollen. In the former, it is called female sterility and in the latter male sterility. Examples of citrus with this type of sterility are Navel sweet oranges [*Citrus sinensis* (L.) Osbeck] and Satsuma mandarins (*Citrus unshiu* Marcovitch). The latter are species of obligate parthenocarpy (Vardi *et al.,* 2008). Not only does Navel orange produce no viable pollen because of the degeneration of pollen mother cells, but it also shows ovule abortion because of degeneration before meiosis (Iwamasa, 1966). Satsuma mandarin has male sterility because four univalents cause irregularities during meiosis, and although some ovules are functional most of them abort (Iwamasa, 1966).

In flowers with homogenetic sterility, pollen and ovules are fertile, but mechanisms of sexual incompatibility, pollen-pistil, make fertilization impossible. If such incompatibility occurs between flowers of the same plant or between plants with the same genotype, it is called self-incompatibility; if it occurs between plants with different genotypes, it is called cross-incompatibility. Homogenetic sterility may be gametophytic or sporophytic type. In the former, pollen is able to germinate on the stigma, but the pollen tube growth is arrested in the style (McClure *et al*., 1990). Clementine mandarins (*Citrus clementina* Hort ex. Tan) and most hybrid like-mandarins have this type of sterility, as the pollen tube stops growing between the stigma and style a few days after pollination (Eti and Stosser, 1992). When incompatibility is sporophyte, the self-incompatibility reaction occurs rapidly between the pollen and stigma epidermal cells surface, pollen being unable to germinate due to the activity of two highly polymorphic proteins encoded by tightly-linked self-recognition genes contained within the S-locus haplotype: the stigma-expressed S-locus receptor kinase, and its pollen-coat localized ligand, the S-locus cysteine-rich protein (Tantikanjanaa *et al.*, 2010). This type of infertility is mainly common in Brassicaceae.

Moreover, cytological sterility is due to chromosomal abnormalities during meiosis of microspores. This is the case of triploids. Obtaining tangerines with this genetic alteration is, at present, a major target for citrus improvement.

Finally, post-fertilization ovule abortion (stenospermocarpy) is due to abnormal meiosis in triploid plants or to defects in endosperm development (Varoquax *et al.,* 2000). In stenospermocarpic fruits, pollination and fertilization occur but both the seed coat and endosperm cease their normal development at early stages, exhibiting quantitative and qualitative variation in the degree of seed development (Striem *et al.,* 1992; Hanaina *et al.,* 2007). Although very uncommon, stenospermocarpy has been observed in a pummelo (*Citrus grandis* Osb.) mutant where parthenocarpic ovary development occurs after the abnormal development of the embryo in the post-zygotic stage, promoting seed abortion (Chai *et al*., 2011), and in the 'Mukaku Kishu' cherry orange fruit (*Citrus kinokuni* hort. ex Tanaka) (Yamasaki *et al.,* 2007; 2009), a species with limited production.

### *2.3. Hormonal control of fruit set*

Plant growth regulators control the flower to fruit transition in citrus. After hormonal induction of ovary growth, fruitlet development critically depends on the nutritional availability i.e. mineral elements and photoassimilates (Guardiola *et al*., 1984; Mehouachi *et al.*, 1995; Agustí, 2003) as well as the sink strength or the ability to produce growth promoting substances such as auxins (Takahashi *et al*., 1975; García-Papi and García-Martinez, 1984) gibberellins (GAs) (García-Papi and García-Martinez, 1984; Sagee and Erner, 1991) and cytokinins (Mauk *et al.*, 1986; Hernández-Miñana and Primo-Millo, 1990; Sagee and Erner, 1991). GAs and cytokinins promote cell division and its application during anthesis promotes ovary development through a stimulus in the transport of photoassimilates and minerals. In seeded varieties self-pollination allows for the transition from flower to fruit development through an increased level of endogenous GAs in the ovary. In these varieties in the absence of self-pollination, the fruit drops from the plant due to its non-parthenocarpic capacity (Garcia-Papi and Garcia-Martinez, 1984; Ben-Cheikh *et al*., 1997). In seedless varieties,

cross-pollination and fertilization also improve fruit set (García-Papi and García-Martinez, 1984), and in the absence of cross-pollination, the transition from flower to fruit development depends on parthenocarpic capacity, so that the higher $GA_1$ content in the ovary during anthesis the higher fruit set (Talón *et al.,* 1992).

The role of auxin in citrus fruit set is unclear. Auxin ovary content increases the days following anthesis significantly decreasing during the next four weeks, and again increasing at 30-35 days of anthesis during early fruitlet development (Takahashi *et al*., 1975; Kojima et al., 1996). In this stage, auxins are related to the abscission process due to polar transport from the fruit to the pedicel, preventing early fruitlet abscission (Else *et al.,* 2004; Blanusa *et al.,* 2005). Indolacetic acid (IAA) is an essential stimulator in vascular tissue differentiation (Aloni, 2010), and a direct effect of exogenous synthetic auxins promoting the development of citrus peduncle vascular tissue was demonstrated (Mesejo *et al.,* 2003). IAA also reduces the sensitivity of cells to ethylene in the abscission layers (Paterson, 2001).

Abscisic acid (ABA) concentration in the ovary increases at petal fall and during the physiological fruit drop correlating the abscission rate (Zacarias *et al.*, 1995; Gómez-Cadenas *et al.,* 2000). But most importantly, ABA mainly increases in fruits with significantly reduced growth rates due to a reduced GA content (Zacarias *et al.,* 1995) or to a reduced availability of carbohydrates (Gómez-Cadenas *et al.,* 2000). The ABA rise leads to an increase in 1-Aminocyclopropane-1-Carboxylic acid concentration, the ethylene precursor, and thus to an increase in ethylene synthesis which in turn activates the abscission zone causing fruitlet drop (Gómez-Cadenas *et al.,* 2000; Agustí *et al.,* 2007 and 2008). Therefore, ABA, like auxin, could have an intermediate role in the abscission process (Estornell *et al.,* 2013).

The aforementioned suggest that hormones cross talk regulates the onset of pharthenocarpic fruit development. However, in *Citrus*, there is evidence that firmly supports the predominant role of GAs, and, in particular, the $GA_1$ ovary concentration at anthesis (Talón et al., 1992).

### *2.4. Gibberellins*

Gibberellins (GAs) are chemical structures that regulate various processes in plants and are part of the group of hormones. The best-known processes regulated by GAs are seed germination, stem growth, induction of flowering, pollen development, fruit set, and fruit growth (Sponsel and Hedden, 2004).

GA was first isolated from the pathogenic fungus *Gibberella fujikuroi* in *1935,* from which they derive their name. The presence of large quantities of GAs as secondary metabolites in this fungus leads to the extensive overgrowth of infected rice plants. Thus, from the time of their discovery, GAs have been known to be effective in promoting stem elongation, and their characterization from the fungus was followed later by their identification as natural components of non-infected plants. In the mid 1950s, gibberellic acid ($GA_3$) was isolated, from the filtrate secreted by the fungus, the inducer compound growth of the stem. Subsequently, its biological activity was demonstrated in plants, since the application of $GA_3$ purified extracts of *Gibberella fujikuroi* over dwarf mutants of corn and pea regained their normal rise (Hedden and Phillips, 2000).

Nowadays, there are 136 fully characterized GAs, designated gibberellin A1 ($GA_1$) through $GA_{136}$. These have been identified from 128 species of vascular plants, and also from seven bacteria and seven fungi (MacMillan and Takahashi, 1968). However, only a few of them contrasted biological activity in some plants, these being $GA_1$, $GA_3$, $GA_4$, $GA_5$, $GA_6$ and $GA_7$ (Fig.2B). The presence or absence of a $\beta$-hydroxyl group at the C-2 position, C-3 and C-13 of *ent*-giberelano of GA C-19 determines the existence of biological activity of the GAs. Thus, $\beta$-hydroxylation at the C-3 seems crucial for biological activity of the GAs, i.e. $GA_1$ (Talón, 2000). In *Citrus* the most important hormone is $GA_1$. On the other hand, when $\beta$-hydroxylation is replaced with other functional groups in the C-2 and C-3, other active GAs, such as $GA_5$ and $GA_6$, are produced. The concentration of bioactive GAs in plants is in the range $10^{-11}$ – $10^{-9}$ g/g fresh weight, depending on the tissue and species, and is closely regulated.

### *2.4.1 Chemical structure*

Gibberellins possess tetracyclic *ent*-gibberellane (C20) or 20-nor-ent-gibberellane (C19) skeletons (Fig 2A). The C20-GAs have the full complement of 20 carbon atoms, whereas the C19-GAs possess only 19 carbon atoms, having lost carbon-20 by metabolism. In almost all the C19 - GAs the carboxyl at C-4 forms a lactone at C-10. Other structural modifications can be made to the *ent*-gibberellane skeleton of both C20- and C19-GAs, such as the insertion of additional functional groups. Further the position and stereochemistry of these substituents can have a profound effect on the biological activity of the GAs (Fig. 2A). For example, a hydroxyl (OH) group in the 3 $\beta$ -position is required for growth-promoting activity (in red figure 2B, $GA_4$, $GA_1$ and $GA_3$), whereas the insertion of an OH in the 2 $\beta$ -position will substantially reduce the bioactivity of an active GA (in green figure 2B for $GA_{110}$, $GA_{51}$, $GA_{34}$, $GA_{97}$, $GA_{29}$ and $GA_8$). In either instance the insertion of an OH in $\alpha$ -orientation has little effect. The C20-GAs do not normally have biological activity *per se*, but can be metabolized to C19-GAs that may be bioactive (Fig. 2B).

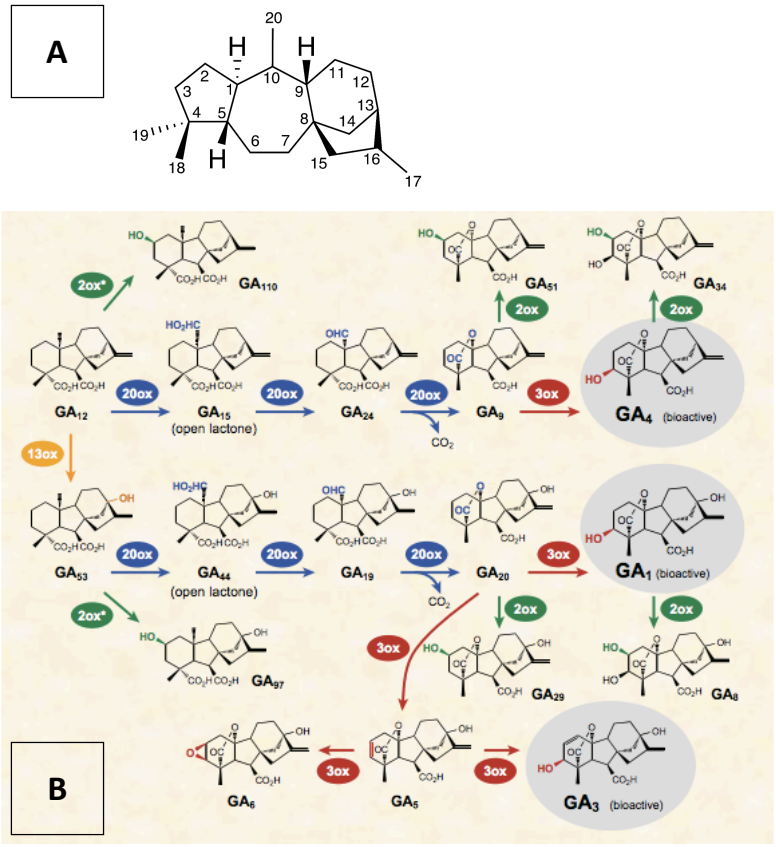



**Figure 2. (A)** Skeleton of ent-gibberellane, basic structure of all gibberellins. **(B)** Biosynthesis pathway of active GAs, $GA_4$, $GA_1$ and $GA_3$. Adapted from Yamaguchi, 2008.

### *2.4.2 Biosynthesis and catabolism*

The first few steps of the pathway, from transgeranylgeranyl diphosphate to $GA_{12}$-aldehyde, are common to all species. The final steps to produce active GAs are species specific, but in most cases they require activity of the GA 20-oxidase (GA20ox) and GA3ox enzymes. By contrast, the enzyme GA2ox impedes GA activity by deactivating GAs. The level of endogenous active GA is governed by feedback regulation, where active GAs suppress the expression of the GA20ox and GA3ox genes and promote the expression of the GA2ox gene. The existence of genes encoding GA deactivating enzymes catalyzing 16 $\alpha$ ,17-epoxidation in rice (Zhu *et al*., 2006) and the formation of GA methyl esters in *Arabidopsis* (Varbanova *et al*., 2007) has been reported although the importance of these reactions for GA homeostasis in other species is unknown.

As diterpenoids, GAs are synthesized from geranylgeranyl diphosphate (GGPP) via isopentenyl diphosphate (IPP), which is the 5-carbon building block for all terpenoid/isoprenoid compounds. In plants the mevalonic acid (MVA) pathway is cytosolic, and IPP formed by this route is further metabolized to sesqui- (C15), and tri-terpenoids (C30), including sterols. In contrast, the MEP pathway is plastidic, and IPP formed in plastids is converted to monoterpenes (C10), diterpenes (C20), including GAs and the phytyl side-chain of chlorophyll, as well as tetraterpenes (C40), including carotenoids. GGPP is, therefore, a key metabolite from which the metabolic pathway branches in several directions, leading to the *ent*-kaurenoids and GAs, the phytyl side-chain of chlorophyll, phytoene and carotenoids, and the nonaprenyl (C45) side-chain of plastoquinone. Thus, a manipulation of one of the branch pathways might have significant effects on the flux through other branches.

The GA-biosynthetic pathway can be divided into three parts. The first part, which occurs in plastids, leads to the synthesis of the tetracyclic hydrocarbon, *ent*-kaurene. In the second part of the pathway, takes place occurs in the endoplasmic reticulum, *ent*-kaurene is sequentially oxidized to yield the first-formed GA, $GA_{12}$ and its 13-hydroxylated analog $GA_{53}$. In the third part of the pathway, which is found in the cytosol, $GA_{12}$ and $GA_{53}$ are further oxidized to other C20-GAs, and C19-GAs.

The first step toward the synthesis of GAs is the conversion of GGPP to *ent*-kaurene. This step allows for a cyclization of the linear GGPP to the tetracyclic *ent*-kaurene, and this occurs in two stages (Fig. 3). GGPP is converted first to the bicyclic compound, *ent*-copalyl diphosphate, by *ent*-copalyl diphosphate synthase (CPS), which was previously called *ent*-kaurene synthase A, and the new cyclization of CPP to *ent*-kaurene is called synthase B.

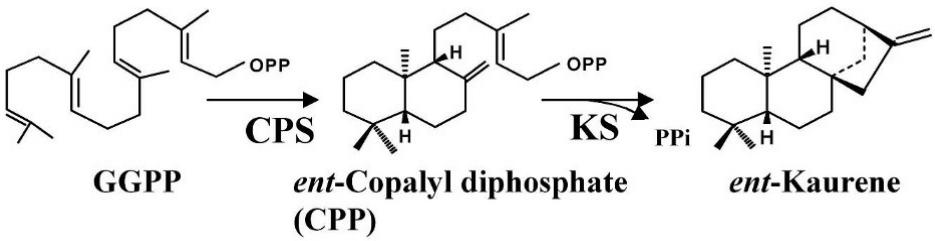



**Figure 3**. Two-step cyclization of GGPP to ent-kaurene via *ent*-copalyl diphosphate, catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS).

This pathway continues with a series of oxidation of *ent*-kaurene. Initially, it is converted by a membrane-associated cytochrome P450 monooxygenase, *ent*-kaurene oxidase (KO), to *ent*-kaurenoic acid, which is oxidized by a second P450, *ent*-kaurenoic acid oxidase (KAO), to $GA_{12}$. The formation of $GA_{12}$-aldheyde from *ent*-kaurene requires six steps, with two enzymes involved, each catalyzing three reactions: KO catalyzes the sequential oxidation of the C-19 methyl group of *ent*-kaurene via the alcohol and aldehyde to the carboxylic acid, while KAO oxidizes C-7 of *ent*-kaurenoic acid to produce ent-7-hydroxykaurenoic acid, which is then oxidized by this enzyme on C-6 to form $GA_{12}$-aldehyde. Finally, KAO oxidizes $GA_{12}$-aldehyde on C-7 to produce GA12. The conversion of ent-7- hydroxykaurenoic acid to $GA_{12}$-aldehyde involves the contraction of ring B from six C atoms to five, transforming the *ent*-kaurane carbon skeleton to the *ent*-gibberellane structure.

At this point, $GA_{12}$-aldehyde lies at a branch-point in the pathway undergoing either oxidation at C-20, or hydroxylation on C-13 to produce $GA_{53}$. $GA_{12}$ and $GA_{53}$ are

precursors to the so-called non-13-hydroxylation and 13-hydroxylation pathways, respectively, and another major pathway is called 3 $\beta$ -hydroxylation (Fig. 4).

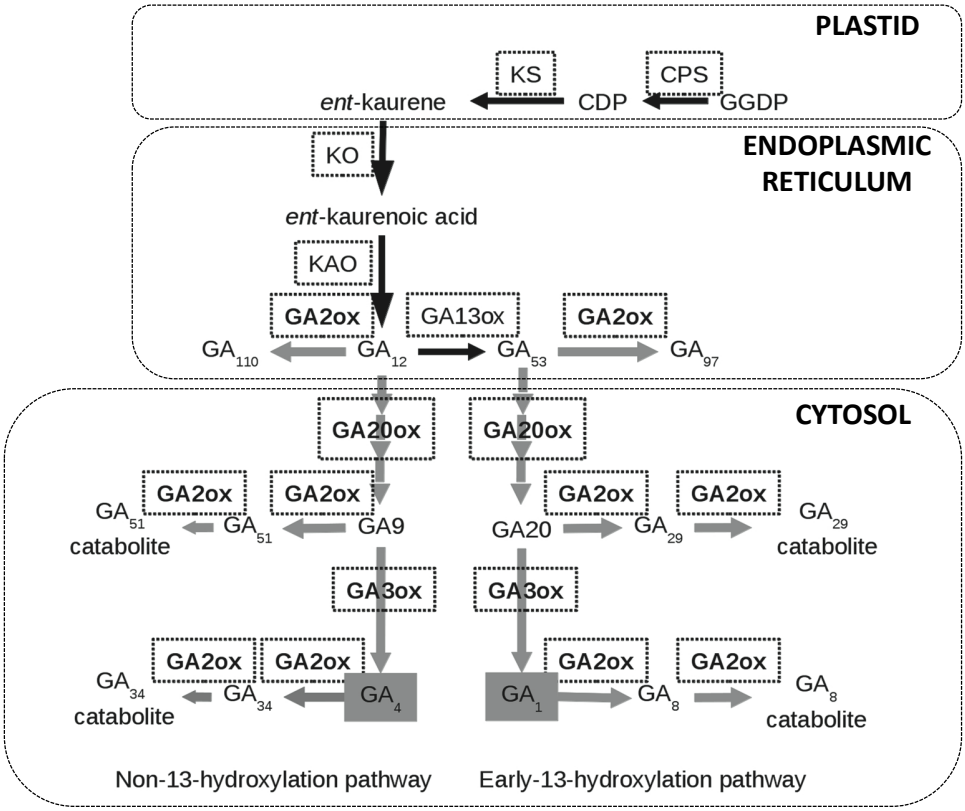



**Figure 4**. Metabolism of $GA_1$ and $GA_4$ in plants. Schematic representation of the early-13-hydroxylation and non-13-hydroxylation pathways in plants. Enzymes are indicated in white boxes. Abbreviations: geranylgeranyl diphospate (GGDP), *ent*-copalyl diphosphate (CDP), *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid synthase (KAO), GA 13-oxidase (GA13ox), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), and GA 2-oxidase (GA2ox).

These reactions are catalyzed by soluble 2-oxoglutarate-dependent dioxygenases. The first enzyme, GA 20-oxidase (GA20ox), is responsible for removing of C-20 in the formation of the C19-GA skeleton. $GA_{12}$ and $GA_{53}$ are converted by this

enzyme in parallel pathways, non-13-hydroxylation and 13-hydroxylation to $GA_9$ and $GA_{20}$, respectively (Fig. 4), by sequential oxidation of C-20 to the alcohol and aldehyde, and then removes of this C atom with formation of the 4, 10-lactone. GA20ox converts each of the C20-GA intermediates in the reaction sequence, although the alcohol intermediate must be present as the free alcohol. These alcohol intermediates form lactones with the 19-carboxyl group when extracted from plant tissues, and are then no longer oxidized by GA20ox. It is unclear whether the -lactones form naturally in plants, but it is of interest that plant tissues, but apparently not seeds, contain an enzyme capable of converting the -lactones to the aldehydes (Ward et *al*., 1997). This enzyme may serve to ensure formation of C19-GAs when GA20ox activity is low, as in plant tissues, when lactone formation may compete with further oxidation of the alcohol intermediate. In most systems, GA20ox forms C20-GAs containing a 20-carboxylic acid group as minor biologically inactive by-products, which are not converted to C19-GAs. However, these tricarboxylic acid GAs are major products of the GA20ox present in endosperm and immature embryos of pumpkin (*C. maxima*) this being the first GA20ox to be cloned (Lange *et al*., 1994). The function of this pumpkin enzyme is considered abnormal as this type of activity has not been encountered in other species or, indeed, in vegetative tissues of pumpkin. The chemical mechanism responsible for the loss of C-20 has not been identified. There is evidence that it is lost as $CO_2$ and that both O atoms in the lactone function originate from the 19-carboxyl group. Direct removal of C-20 as $CO_2$ requires the formation of an intermediate between the aldehyde and final C19-GA product, but none has been identified and it may remain bound to the enzyme.

The growth-active GAs, $GA_4$ and $GA_1$, are formed by 3-hydroxylation of $GA_9$ and $GA_{20}$, non-13-hydroxylation and 13-hydroxylation pathways, respectively, catalyzed by GA 3-oxidases (GA3ox) (Fig. 4). For example, oxidation of both C-2 and C-3 produces a 2, 3-double bond, as in the conversion of $GA_{20}$ to $GA_5$. Further oxidation of $GA_5$, initially on C-1 and then on C-3, by the same enzyme results in the formation of $GA_3$ (Albone *et al*., 1990; Spray *et al*., 1996). While most GA3-oxidases are specific for C19-GAs, some plants, and particularly seeds, produce 3-hydroxylated C20-GAs.

A third class of dioxygenase, GA 2-oxidase (GA2ox) is responsible for the irreversible deactivation of GAs by 2-hydroxylation, so ensuring GA turnover, which is

necessary for effective regulation of GA concentrations. In some tissues, such as the cotyledons and, particularly, the testae of developing pea seeds, C-2 is oxidized further to the ketone, giving rise to the so-called GA-catabolites (Fig. 4). Studies of GA2ox function using recombinant protein prepared in *E. coli* reveal that these enzymes are capable of both reactions 2- hydroxylation and ketone formation (Thomas *et al*., 1999). Most 2-oxidases are specific for C19-GAs, and will accept the 3-hydroxy bioactive GAs and their non-3-hydroxylated precursors as substrates, and form group (I) and (II) in this sub-family. C20-GAs may also be 2-hydroxylated, and *Arabidopsis* and *Vitis vinifera* have been shown to contain GA 2-oxidases that are specific for these compounds (Schomburg *et al*., 2003; Giacomelli *et al.,* 2013) and form group (III). Such enzymes may be essential to maintain of GA homeostasis when levels of C20-GA precursors become very high.

### *2.4.3. Gibberellins, cell division and parthenocarpy*

As early as the 1960s, studies reported the effect of exogenous applications of $GA_3$ on promoting fruit set in low-parthenocarpic varieties, the Clementine mandarin (*C. clementina* Hort. *ex* Tan) (Soost and Burnett, 1961; Rivero *et al*., 1968), or specific sweet orange cultivars (*C. sinensis* L. Osb) (Agustí *et al*., 1982).

Later work in the 1990s highlighted the decisive role of endogenous bioactive, in parthenocarpic fruit development, GA concentration being much higher in the obligate parthenocarpic Satsuma mandarin (*C. unshiu* Marc.) than in the Clementine mandarin. Specifically, both mandarins present the early 13-hydroxylation pathway and the non-hydroxylation pathway but, at anthesis, the concentration of $GA_1$ increases twofold in the Satsuma compared to the Clementine ovary, this difference being maintained up to 5-7 days after the petal fall stage. On the other hand, $GA_8$ increases in the Clementine ovary (Talón *et al*., 1992). However, the molecular factors that regulate $GA_1$ synthesis and reactivation during natural parthenocarpic fruit development in *Citrus* are unknown.

On the other hand, it is generally assumed that ovary growth and cell division are temporally reduced during the period of anthesis until pollination and fertilization occur, and that the increase in GA in the ovary may be part of the hormonal stimuli

that reactivates cell division triggering fruit development (Gillapsi *et al*., 1993). However, in seeded *Citrus*, this was not confirmed, since pollinated and un-pollinated ovaries grow in a similar manner and both have a similar rates of cell division and enlargement in the pericarp, but different GA concentrations (Ben-Cheikh *et al*., 1997). The mechanism allowing GA-responsive ovary growth by cell division activation is not well defined. The role of GA regulating cell proliferation was reported years ago in the context of shoot elongation of the herbaceous plant *Silene armenia*, the data indicating a spatial correlation between the accumulation of $GA_1$ and the enhanced mitotic activity that occurs in the subapical meristem (Talón *et al.,* 1991). In *Citrus*, the shorter and longer internodes produced by antisense and sense expression of *CcGA20ox1*, respectively, seem to be due to repression and induction of cell division rather than cell elongation (Fagoaga *et al*., 2007). The basic cell cycle machinery is composed by two key classes of regulatory molecules namely cyclins (CYCs) and cyclin-dependent kinases (CDKs). While A-type CDKs are constitutively expressed during the cell cycle, B-type CDKs are plant specific and are involved in regulating G2/M phase progression; A, B and D-type CYCs regulate the S and/or G2/M phases (Inze and de Veyler, 2006). Auxins and cytokinins have been proposed to regulate several steps from the cell cycle machinery, (Inze and de Veyler, 2006; Scofield et al., 2014), but the possible roles of GA in cell cycle progression are just beginning to be addressed, and although experiments with monocots and annual plants have analysed the relationship between GA and cell division (Fabian *et al.,* 2000; Asahina *et al.,* 2002), specifically in the context of leaf growth (Nelissen *et al.,* 2012) and root growth (Ubeda-Tomás *et al.,* 2009). However, little is known about the relationship between GA, cell division and parthenocarpy.

## 3. HYPOTHESIS AND OBJECTIVES

### *3.1 Hypothesis*

Although we know precisely the time-course of GA content in the citrus ovary the days around anthesis and its correlation with fruit set, it is not known with precision:

1. The molecular factors that regulate $GA_1$ concentration in the ovary that determine fruit set. Are differences between varieties determined by $GA_1$ synthesis, $GA_1$ deactivation to $GA_8$ or both?
2. The specific site of GA synthesis and its spatial variation inside the parthenocarpic ovary. While in seeded varieties hormone synthesis that occurs in fertilized ovules is the primary stimulus controlling early fruit development, is there any specific tissue-dependent regulation of parthenocarpic fruit set?
3. The specific role of GA regulating the fruit set process. Is GA in the ovary a direct regulator or an indirect part of the hormonal stimuli that reactivates and maintains cell division triggering fruit set?
4. While initiation of GA synthesis is thought to be autonomous in the sterile parthenocarpic species, it is unclear whether it is autonomous or stimulated in the fertile self-incompatible species, which present a wide variation in their parthenocarpic ability.

Attending to the aforementioned, in this PhD Thesis, the following hypothesis was tested:

**The autonomous synthesis of gibberellins in the pericarp directly activates and maintains cell division in the ovary, promoting the flower to fruit transition in parthenocarpic citrus species.**

### *3.2. Objectives*

To test this hypothesis the following three objectives were established, and experiments were carried out using 5 *Citrus* cultivars with different characteristics regarding their sexual behaviour and parthenocarpic ability: *Citrus sinensis* cvs. 'Navel Barnfield' and 'Pineapple' are sterile and fertile self-compatible oranges, respectively. 'Clemenules' and 'Marisol' mandarins (*Citrus clementina* Hort. ex Tan) are fertile self-incompatible cultivars differing in their parthenocarpic ability, low and high, respectively. Satsuma 'Owari' mandarin (*Citrus unshiu* Marc.) is a sterile cultivar with high parthenocarpic ability.

1. To identify the specific tissue where GAs biosynthesis occurs during fruit set in seeded ('Pineapple' sweet orange) and parthenocarpic ('Navel Barnfield' sweet orange) fruits.
2. To correlate GAs biosynthesis and endogenous concentration with the spatial distribution of cell division and its temporal variation in the ovary during parthenocarpic fruit set in Satsuma and Clementine species, which differ in their parthenocarpic ability.
3. To analyse the effect of self-pollination on $GA_1$ and $GA_4$ synthesis in the ovary during the parthenocarpic fruit set of two self-incompatible Clementine mandarins, 'Clemenules' and 'Marisol', which differ in their parthenocarpic ability.

# MATERIALS AND METHODS

## 1. Plant material and experimental design

***<u>Experiment I.</u> GA biosynthesis location during fruit set in seeded and parthenocarpic citrus fruit***

The plant material for this study involved 12-year-old *Citrus sinensis* (L.) trees cvs. Barnfield and Pineapple grafted onto Carrizo citrange rootstock (*Poncirus trifoliate* Raff. X *C. sinensis* (L.) Osb.), planted 5m x 5m apart. 'Navel Barnfield' orange is a species of obligate parthenocarpy, presenting 1) no viable pollen due to the degeneration of pollen mother and 2) marked ovule abortion because of degeneration before meiosis. Therefore, this cultivar is always seedless. On the other hand, 'Pineapple' orange is proterandous and cleistogamic, which permits abundant self-pollination because anthers begin to shed pollen while they are still pressed against the stigma in the closed or opening flower, allowing for pollination before anthesis. This genotype was selected because of its absolute requirement for pollination to develop fruits, its elevated pollen viability, and its high seed production under natural conditions.

Experimental fields were located in the IVIA Research Station (Moncada, Valencia, Spain), and trees were grown using standard cultivation practices. Fifteen trees per cultivar were selected in spring (2013) for their uniformity in flowering, size and vigour. The experiment was performed with 1000 terminal flowers of cv. Barnfield and of cv. Pineapple. Of those, 100 flowers per cultivar randomly distributed in the trees were used for fruit set evaluation at the end of the physiological fruit drop; 50 flowers from 'Pineapple' orange were sampled from preanthesis until petal fall to monitor pollen tube development in the pistil and the timing of ovule fertilization by fluorescent microscopy; 100 flowers per cultivar were sampled at petal fall for GA concentration analysis by UPLC-MS/MS; 600 flowers per cultivar were sampled at petal fall for RNA extraction and RT-PCR analysis for the study of GA-oxidases gene expression (for details see RT-PCR analysis section); finally, 100 flowers per cultivar were sampled at petal fall to study the specific site of GA synthesis by the *in situ* hybridization of *GA20ox2* transcripts.

***Experiment II.* Constitutive activation of cell division, GA biosynthesis and parthenocarpy**

The plant material for this study involved adult Clementine mandarin trees cv. Clemenules (*Citrus reticulata Hort*. ex. Tanaka) and adult Satsuma mandarin trees cv. Owari (*Citrus unshiu Mak* Marc.) both grafted onto *Citrus aurantium* rootstock in a commercial orchard located in Menfi (Sicily, Italy, in 2012), or grafted onto Carrizo citrange roostock in a commercial orchard located in Líria (Valencia, Spain, in 2013). These mandarins show low and high tendency to develop parthenocarpic fruits, respectively. Trees were grown using standard cultivation practices in both orchards. Five trees per cultivar were selected for the experiment, which was repeated during 2 consecutive seasons (2012-13). The experiment was performed with 4,000 flowers of cv. Clemenules and 3,000 of cv. Owari. Of those, 300 flowers per cultivar randomly distributed in the 5 trees were used for fruit set and fruit diameter evaluation without detaching them from the tree; 100 flowers per cultivar were used for fruitlet fresh weight evaluation; 100 flowers per cultivar were used for histological procedures in which cell division rate and *GA20ox2 in situ* hybridization were determined; finally, 2,900 flowers of cv. Clemenules and 1,700 of cv. Owari were sampled for RNA extraction and RT-PCR analysis to study GA-oxidases and CycA1,1 gene expression (for details see RT-PCR analysis section). The difference in the number of flowers between the cultivars is attributed to the size and weight of the young ovaries, which determines the quantity for extraction of RNA. On tree measurements and sampling were conducted every 5 days, from 10 days before anthesis until 15 days after anthesis. To avoid cross-pollination and fertilization the flowers were bagged.

***Experiment III.* Exogenous regulation of cell division and parthenocarpy**

This experiment involved adult trees of Clementine cv. Clemenules and Satsuma cv. Owari mandarins from the same commercial orchard located in Líria (Valencia, Spain). Five trees per cultivar, different from those selected in the experiment II (2013), were selected in spring 2014. To determine the effects of gibberellic acid ($GA_3$) and paclobutrazol (PBZ) on cell division and parthenocarpy, 600

flowers per cultivar were treated at anthesis with 5 ml flower$^{-1}$, using $GA_3$ (10 mg l$^{-1}$) and PBZ (1000 mg l$^{-1}$) as a foliar spray. The same number of control flowers was selected for comparison. Of those, 100 flowers per treatment were used for fruit fresh weight evaluation during 4 weeks; 100 flowers per treatment were sampled every other week to determine the rate of cell division; the other 400 flowers were sampled at 6 hours, 2 days and 7 days after treatment to determine GA concentrations, and gene expression analysis (*CycA1,1* and *GA-oxidases*) by RT-PCR analysis. To avoid cross-pollination and fertilization, the flowers were bagged.

***Experiment IV. In the self-incompatible Clementine mandarin, Is GA synthesis autonomous or boosted by self-pollination?***

Adult Clementine mandarin trees cvs. Marisol and Clemenules (*C. clementina* Hort. ex Tan.), grafted onto Carrizo citrange rootstock (*Poncirus trifoliata* Raff.×*C. sinensis* (L.) Osb.), were used in the fourth experiment. These mandarins show high and low tendency to develop parthenocarpic fruits, respectively. Trees were grown in the commercial orchard located in Líria (Spain). Fifteen trees per cultivar were randomly selected in spring 2012. The experiment was performed with 2000 flowers per cultivar. The same number of flowers were self-pollinated and bagged or emasculated and bagged (control) for comparison. Of those, 400 flowers were used for fruit set and diameter evaluation without detaching them from the tree; fruit set was recorded weekly for 9 weeks after pollination and final fruit yield was determined by counting all the fruits at harvest. The diameters of persisting fruitlets were measured at 4-day intervals during the 5 weeks following pollination; 200 flowers were sampled for histological analysis, and 3400 flowers were sampled for carbohydrate and hormone (GA, IAA, ABA) analyses. Carbohydrate contents were determined in fruitlets at 0, 5, 16 and 23 DAP; thirty fruitlets per treatment and date were collected and samples were frozen immediately in liquid nitrogen, lyophilized and stored as powder at −28◦ C. ABA and AIA were determined in 30 ovaries per treatment collected at 0, 5, 16 and 23 DAP, and GA content was determined in 150 ovaries per treatment collected

at 0, 5, and 9 DAP. All samples were frozen immediately in liquid nitrogen, lyophilized and stored as powder at −80◦ C.

## 2. Methods

### *2.1 Self-Pollination procedure*

Open flowers of 'Clemenules' and 'Marisol' mandarins at a growth stage 61 on the Citrus Biologische Bundesanstalt, Bundessortenamt and Chemical industry (BBCH)-phenological scale (Agusti *et al.,* 1997) were randomly selected for pollen collection the day before pollination. Anthers were removed and placed at room temperature on a piece of filter paper to dry and trigger dehiscence. Just before anthesis (stage 59-BBCH), flowers from cvs. Clemenules and 'Marisol' (Experiment IV) were self-pollinated by hand and bagged to avoid free pollination. Additionally, 150 flowers from cv. Clemenules were emasculated and bagged to use as control. Since early fruit set is highly dependent on the type of inflorescence (Guardiola *et al*., 1984), only single flowered leafy shoots randomly distributed in the canopy were used for the experiment. Pollination was performed using a small brush.

### *2.2 Fruit set, fruit growth and yield evaluation*

The total number of flowers, on a tree basis, was estimated by extrapolating the mean number of flowers per frame to the total tree surface area. Tree height (h) and canopy radius (r = average of radius in the N, S, E and W directions) were measured. The canopy surface area ($m^2$) was calculated according to the formula of Serfontein and Catling (1968), assuming the shape of a prolate spheroid $SA = \pi r^2 + \pi(hr/f)\sin^{-1} f$ were $f = (1 - b^2/a^2)^{1/2}$.

Fruit set was calculated as the percentage of fruits remaining in each tree. Diameters of persisting fruitlets were measured with a digital caliper (Mitutoyo, USA) without detaching them from the tree, results being expressed in mm. Fruitlet fresh weight was both directly measured in sampled fruitlets with a precision weighing

balance and calculated by means of a regression analysis, regression curves being calculated by weighing and measuring the diameter of each fruitlet of 50 fruitlets harvested at random from the experimental control trees in the same orchard. Final fruit yield was determined by counting all the fruits at harvest. The absence of seeds was confirmed in mature fruits.

### *2.3 Microscopic studies*

#### ***Histological measurements***

Sampled flowers were fixed in FAA (10% formaldehyde, 10% acetic acid and 80% ethanol at 70%) in the field and transported at 4ºC to the laboratory.

*Pollen grain germination, pollen tube growth and ovule abortion*

Pollen tubes in the stigma and style, as well as ovules, were monitored on squash preparations of pistils previously softened in 5% sodium sulphite in a microwave for 1.5 min, stained with 0.1% aniline blue in 0.1N $PO_4K_3$ (Linskens and Esser, 1957), and observed under a fluorescence microscope (Olympus BX50, Tokyo, Japan) equipped with a U-MWU filter (Olympus). The percentage of pollen germination on the stigma was calculated by counting 200 pollen grains per flower. Pollen tube development in the style was determined as the percentage of the style traversed by the longest pollen tube in each flower (Mesejo *et al.,* 2006). Ovule degeneration was calculated in 20 ovules per flower by counting the number of ovules with a callose layer at the chalazal end (Rodrigo and Herrero, 1998; Rosellini *et al*., 2003; Mesejo *et al.,* 2006).

*Ovary fixation, embedding and sectioning*

Samples of ovaries and pollinated ovaries at different growth stages, were fixed in FAA, and conserved at -20°C until the next step of dehydration. Dehydrated in a tertiary butyl alcohol series (70, 85, 95 and 100%, v/v) and embedded in paraffin with an Automatic tissue processor (Leica TP 1020) and Inclusion Station (Leica EG1150H).

Paraffin-embedded material was sectioned at 6-8 mm in a rotary microtome (Microm HM330) and the sections were attached to Polysine slides (Menzel-Glaser). The sections obtained were rehydrated (3 washes in Histoclear [CellPath, Hemel, United Kingdom], one each in Histoclear:ethanol [1:1, v/v] and an ethanol series [100, 70 and 40%, v/v]).

*Analysis of cell division and morphological parameters of ovaries*

Ovary size was also measured in the paraffin sections to study the relationship between ovary size and cell division. Cell number and size were determined in the same sections. Thus, the preparations were washed in distilled water, stained with methylene blue. Cells were counted along the cross-section of the ovary and mean individual cell size was determined by dividing ovary width by cell number.

The ovary wall width and cell number were measured on 5 cross-sectioned ovaries for each treatment. Cross-sectional cell areas were measured on 100 cells in 5 ovary walls for each treatment. Ovary size was recorded as the width of the ovary wall in its central section.

Preparations were observed and photographed with a bright field microscope (E600, NIKON). The images collected using a photographic camera (NIKON digital) attached to the microscope and processed using a Quantiment 570 Image Analysis System (Leica Cambridge, Cambridge, United Kingdom).

*Scanning electron microscopy (Cryo-SEM)*

To examine specific cell division in the endocarp and juice sacs by cryo-SEM, small pieces of growing ovaries, approximately 0.125 $cm^3$, were excised with a razor blade, immediately frozen in liquid nitrogen (−210°C) and stored at −80°C. At the time of the analysis, samples were mounted, avoiding defrost, on SEM stubs, immediately frozen in nitrogen slush (−210°C) and attached to the specimen holder of a CT-1000c Cryo-transfer system (Oxford Instruments, Oxford, United Kingdom) interfaced with a JSM-5410 (JEOL, Kyoto, Japan; scanning electron microscope (SEM)).

The frozen sample was transferred to the prechamber of the cryo-transfer system, maintained at approximately −130°C and then freeze-fractured with a microtome blade cooled to −170°C. After that, the mounted-sample was first transferred to the sample stage, where the condensed surface water was sublimed by controlled warming to −90°C over 15 min, and afterwards it was transferred again to the cryo-stage and, under vacuum conditions, coated with a film of gold in a Polaron E-6100 sputter coater. SEM was performed at 10–15 kV accelerating voltage, and the cold stage was maintained at a temperature of −130°C. At least 2–3 samples for each species and collecting date (-5, 0 and 10 DAA) were evaluated.

### *2.4 Gene expression analysis by qRT-PCR*

Total RNA was extracted from frozen tissues and subsequently treated with DNase I (RNase-Free DNase Set, Qiagen, USA). The amount of RNA was measured by spectrophotometric analysis (NanoDrop ND-1000 spectrophotometer, Thermo Fisher, USA). The absence of DNA contamination was checked by performing a no-reverse transcription assay which consisted of a PCR with each RNA sample using the Citrus actin primers (Supp. Table 1). No amplified products were detected, which confirmed the purity of the RNA extracts. The transcripts present in 1 µg of total RNA were reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) in a total volume of 20 µl. A 2.5 µl aliquot of a 4-time diluted first-strand cDNA was used for each amplification reaction. Quantitative real-time PCR was carried out on a Rotor Gene Q 5-Plex (Qiagen, USA) using the QuantiTect® SYBR® Green PCR Kit (Qiagen, USA). The reaction mix and conditions followed the manufacturer's instructions with certain modifications. The PCR mix contained 2.5 µl of diluted cDNA, 12.5 µl of QuantiTect® SYBR Green PCR Master Mix (Qiagen, USA), 1.5 µl of 0.3 µM primer F, and 1.5 µl of 0.3 µM primer R, the final volume being 25 µl. The cycling protocol for the amplification consisted of 15 min at 95ºC for pre-incubation, then 40 cycles of 15 s at 94ºC for denaturation, 30 s at 60ºC for annealing and 30s at 72ºC for extension. RT-PCR reactions were repeated three times for each gene and monitored in real time with the Rotor Gene Detector. After amplification, melting-curve analysis excluded

artefactual amplifications. The relative expression of RNA transcripts was quantified with the threshold cycle values (Ct) obtained from each sample using the $2^{-DDCt}$ method (Livak & Schmittgen, 2001). Expression levels were calculated relative to the constitutively expressed ACT-2 and β-LCY genes (Rodrigo *et al*., 2004; Alquezar *et al.*, 2009; Mafra *et al*., 2012). The relative gene expression level is given by $2^{-DDCt}$. Normalization was performed to the first sample date for each species. Two or three independent biological samples under each experimental condition were evaluated in technical triplicates.

Table S1. Primer sequence used in RT-PCR amplification reactions

| | EST code* | Forward primer (5' – 3')<br>Reverse primer (5' - 3') | Predicted product (bp) |
|---|---|---|---|
| LYCOPEN | | GCTCTAGCCTTGTAGGAAAGCCATGG<br>GCGAATTCCGTGTGCACCTTAATCTGTATC | (Alquézar et al., 2009) |
| GA20ox1 | Ciclev10005157m | ACCAAGTGGGTGGTCTTCAG<br>TGAAGGTGTCGCCAATGTTA | 96 |
| GA20ox2 | Ciclev10020694m | GGTGACACCTCCGAACAACT<br>AATGCGTTGAGGGTTTTCAC | 126 |
| | PROBE | GGATTGTTACAATTCAAGCATATTGCC<br>CGACGACGGTTGAAGAGTTC | 476 |
| GA3ox1 | Ciclev10027153m | CAACGCAAGATGTCAAATGG<br>CAGGCCGGGTAGTAATTCAA | 85 |
| GA3ox2 | Ciclev10010629m | CCTGTTGATGGTGCCCTAGT<br>CCGCTTTTGATTCACAGACA | 105 |
| GA2ox1 | Ciclev10021090m | TCCATTTCCAGGCTTGAATC<br>ACATCACCATTCCGTCCAAT | 125 |
| GA2ox2 | Ciclev10024433m | GAAACATCGGCTTCAATGGT<br>GCGCGACTGAATTTTAAAGG | 121 |
| GA2ox3 | Ciclev10015817m | ACACATCTGGCCTCCAAATC<br>ATGGGACTGAAACCCAAGTG | 54 |
| GA2ox4 | Ciclev10005385m | CAAGCCAGTCGAGAATGTCA<br>GAGAAGGCCTGTTGGTTGTC | 99 |
| CYCA1,1 | Ciclev10003984m | CCAGTTTTGTGCAACCATTG<br>TGGACCCTCTCCATGAAGTC | 105 |

Sequence analysis and phylogenetic trees

*GA-oxidase* and *CycA1,1* amino acid sequences of *A. thaliana* and other annual plant species and woody evergreen and deciduous tree species were obtained from the NCBI database (www.ncbi.nlm.nih.gov). Sequences were aligned against the *Citrus clementina* genome using the TBLASTN tool of Phytozome v9.1 database (www.phytozome.net). Based on this sequence similarity, 17 putative homolog GA oxidases were identified in the *Citrus clementina* genome. The GA20ox study was based on the similarity to the characterized amino acid sequences of *CcGA20ox1* and *CcGA20ox2* from the citrus hybrid citrange Carrizo [(*Poncirus trifoliata* Raff. X *Citrus sinensis* (L.) Osb.)]. The GA3ox and GA2ox study was based on the similarity to the proteins *AtGA3ox* 1-4 and *AtGA2ox* 1-8 from *A. thaliana*. Phylogenetic trees are given in Supplementary figures at the end of this chapter. Primers used for qRT-PCR analysis (http://frodo.wi.mit.edu/) are listed in Supplementary Table 1.

### *2.5 Hormone isolation, purification and quantification*

Frozen material in liquid nitrogen was ground into fine powder. Aliquots (about 50 mg fresh weight) of material were extracted with 80% methanol containing 1% acetic acid. Internal standards were added and mixed with the aliquots at 4°C for 1 hour. The internal standards for quantification of each of the different plant hormones were the deuterium-labelled hormones, except for JA, for which dhJA was used. The extraction protocol used is that described in Seo *et al*., (2011) with certain modifications. In brief, for desalination, the extracts were passed through reverse phase columns HLB (Waters). The plant hormones were eluted by 80% methanol containing 1% acetic acid and consecutively applied to cation exchange MCX columns (Waters). The fraction containing the acidic ABA, GAs, IAA was applied through ion exchange WAX columns (Waters). The final residue was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an auto sampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 14 min. The hormones were analysed with a

Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs.

### *2.6* In situ *hybridization*

A single template for *GA20ox2* was generated with a fragment of 476 bp of region of cDNA. The fragment was cloned into the pGem-Teasy vector (Promega), and sense and antisense probes were synthesized using the SP6 and T7 RNA polymerase. Control experiments were performed with antisense probes of *GA20ox2* and no significant signal was detected.

Satsuma and Clementine ovaries at anthesis and 30d later were fixed in FAA (70% ethanol: glacial acetic acid: formalin [18:1:1, v/v/v]), dehydrated in a tertiary butyl alcohol series up to 70% v/v and stored at -20°C until the next step of dehydration. Dehydration was continued (70, 85, 95 and 100%, v/v) and the samples were embedded in paraffin. Paraffin-embedded (described above in ovary fixation) material was sectioned at 6-8 mm in a Microm HM330 rotary microtome and prior to staining, the sections obtained were rehydrated (3 washes in Histoclear [CellPath, Hemel, UK], one each in Histoclear:ethanol [1:1, v/v] and an ethanol series [100, 70 and 40%, v/v]). The 6-8mm thick sections were attached to Polysine slides (Menzel-Glaser). The procedure of *in situ* hybridization was essentially the same as the method developed by Jackson (1992). Digoxigenin-labeled antisense (negative control) and sense RNA probes were synthesised using T7 and SP6 RNA polymerase. The probes corresponding to Sal I – Sac II fragment of GA20ox2 cDNA were used in all *in situ* experiments. After hybridization, slides were washed twice for 1 h in 2×SSC, once for 1 h in 1×SSC and 30 min in 0.5×SSC. The digoxigenin labeled probe was detected with anti-digoxigenin alkaline phosphatase antibody diluted 10:1000 and using NBT/BCIP as a substrate. The reaction was completed with TE buffer; the slides were than viewed and photographed with a bright field microscope. In control *in situ* hybridization, using the sense probe, the alkaline phosphatase activity was at the background level.

### *2.7 Carbohydrate analysis*

The procedure for carbohydrate determination was that described previously by Martínez-Fuentes *et al.* (2010). Briefly, 100 mg of powdered samples were extracted with 1 ml of 800 ml $l^{-1}$ ethanol and purified sequentially using cation and anion exchange columns. The eluates were then passed through a C18 Sep-Pak cartridge (Waters-Millipore, Billerica, MA) and analysed in a Spectra high performance liquid chromatography (HPLC) System (Spectra, San Jose, CA) equipped with a vacuum pump (Spectra P2000, Spectra) and a differential refractometer (Spectra R150, Spectra). Sucrose, glucose and fructose were identified according to their retention times. Results were expressed as milligram per gram dry weight (DW). Starch levels were determined in the pellets that remained after the extraction of soluble sugars. The residue was incubated by shaking for 2 h at 55°C with 0.2 ml of 60 mg $ml^{-1}$ fucose (internal standard) (Sigma Chemica Co. Inc., Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), 0.5 ml of sodium acetate (pH 4.5) and 1 ml of 1218 U amyloglucosidase from Rhizopus (Sigma Chemica Co. Inc.). The glucose released was determined by HPLC as above. Results were expressed as milligram glucose released per gram DW.

### **2.8** ***Statistical analysis***

Parameters were statistically tested by analyses of variance (ANOVA), using the least significant differences (LSD) test for means separation. The experimental data were analysed with Statgraphics Plus 5.1 software (Statistical Graphics, Englewood Cliffs, NJ).

## 2.9 Supplementary figures. Phylogenetic trees.

**Figure S1**. Phylogenetic analysis of GA20 oxidases in *C. clementina*. The tree is a detailed magnification of the most specific region of the maximum tree of a larger alignment (see below). Two ESTs were found in the *C. clementina* genome. The ESTs selected for gene expression studies are Ciclev10020694m, named *GA20ox2,* and Ciclev10005157m, named *GA20ox1*, according to their similarity with citrange Carrizo.

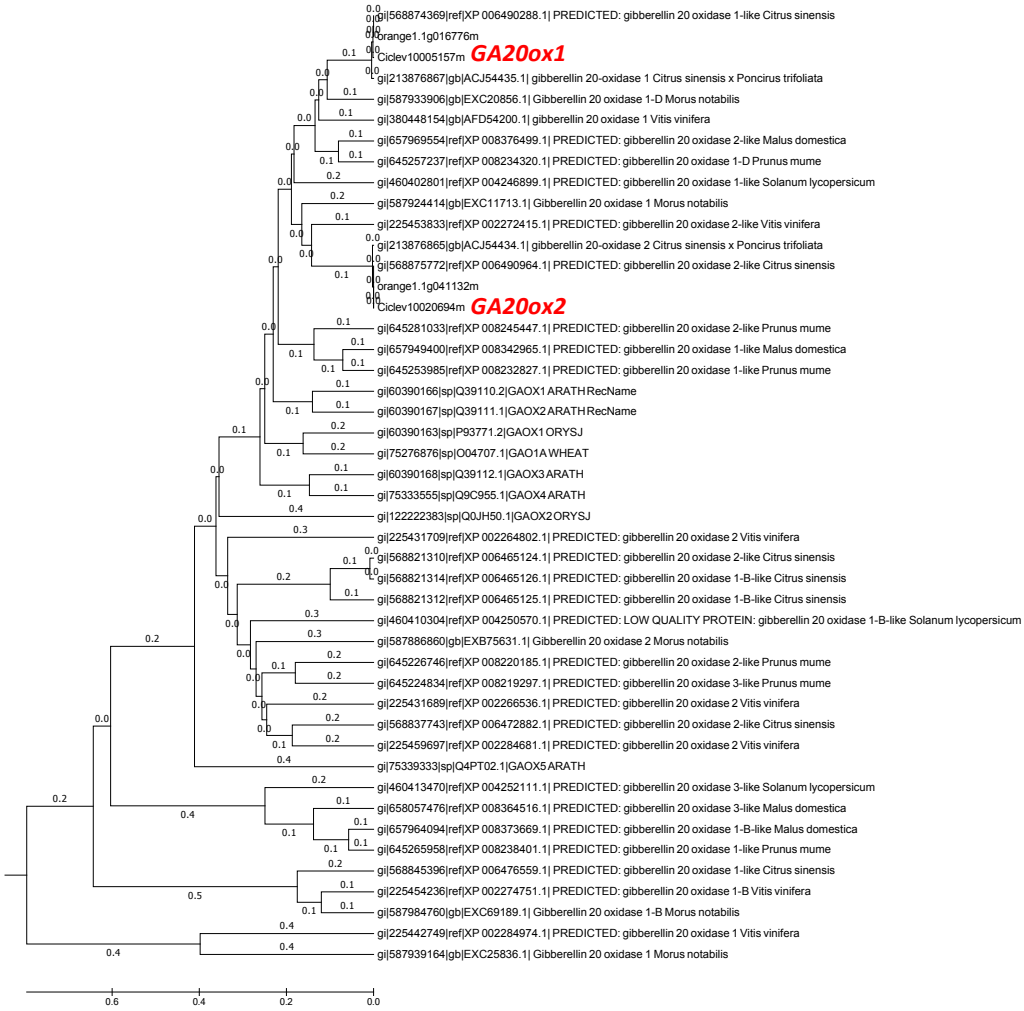


**Figure S2**. Phylogenetic analysis of GA3 oxidases in *C. clementina*. The tree is a detailed magnification of the most specific region of the maximum tree of a larger alignment (see below). Five ESTs were found in the *C. clementina* genome. The ESTs selected for gene expression studies are Ciclev10027153m, named *GA3ox1* and Ciclev10010629m, named *GA3ox2*. ESTs were selected according to their specific function and site of expression during fruit set in *A. thaliana* (Mitchum *et al.,* 2006) and *V. vinifera* (Giacomelli *et al*., 2013).

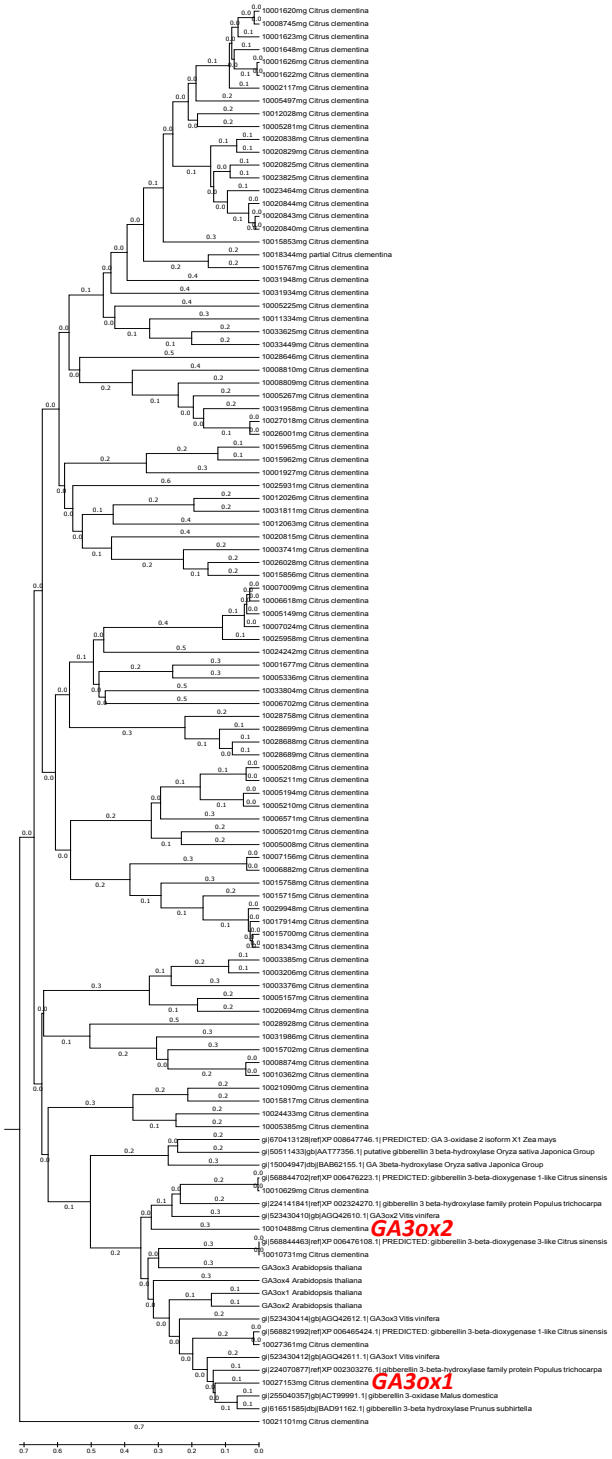


**Figure S3**. Phylogenetic analysis of GA2 oxidases in *C. clementina*. The tree is a detailed magnification of the most specific region of the maximum tree of a larger alignment (see below). Ten ESTs were found in the *C. clementina* genome. The ESTs selected for gene expression studies are Ciclev10021090mg, named *GA2ox1*, Ciclev10024433mg, named *GA2ox2,* Ciclev10015187mg, named *GA2ox3* and Ciclev10005385mg, named *GA2ox4*. ESTs were selected according to their specific function and site of expression during fruit set in *A. thaliana* (Mitchum *et al.,* 2006) and *V. vinifera* (Giacomelli *et al.,* 2013).

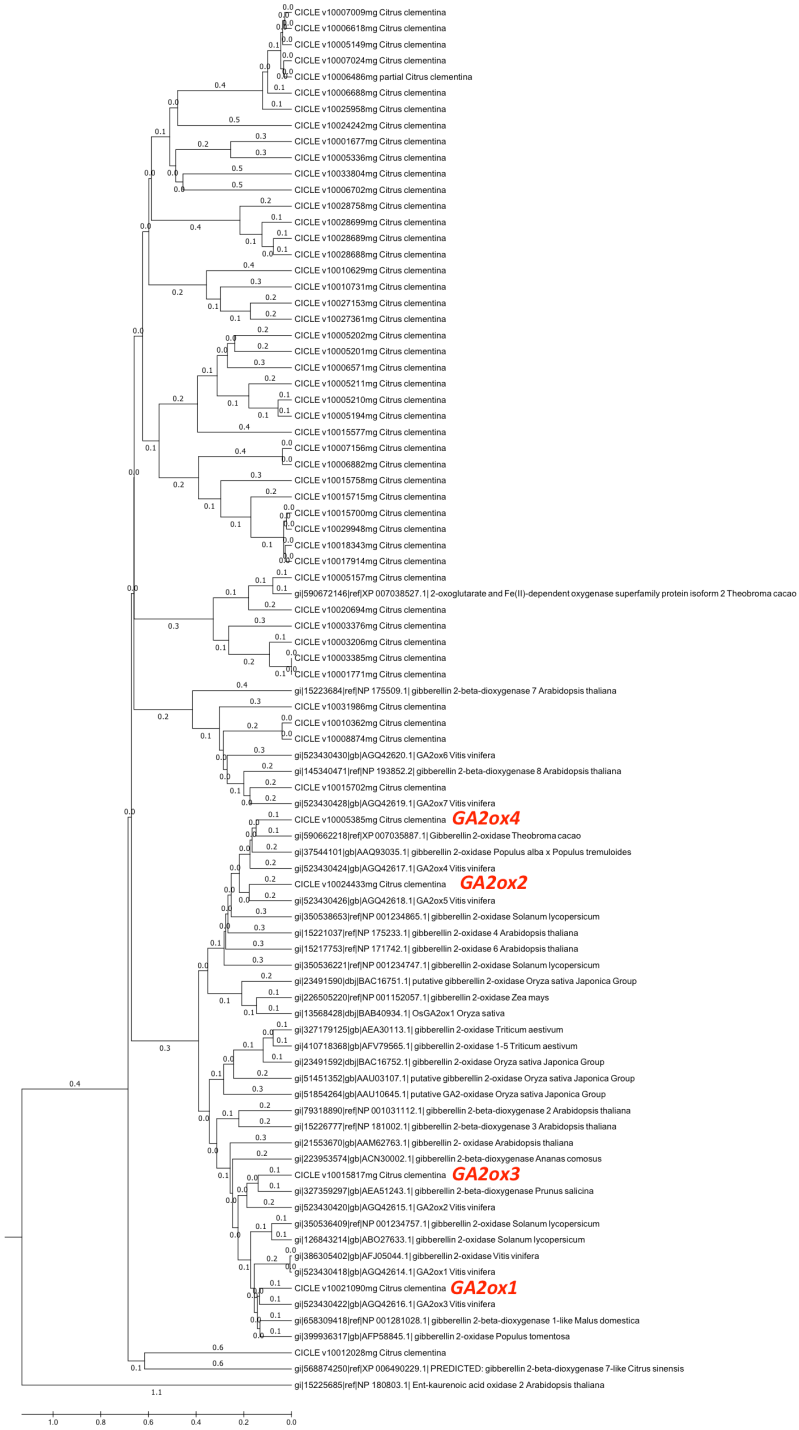
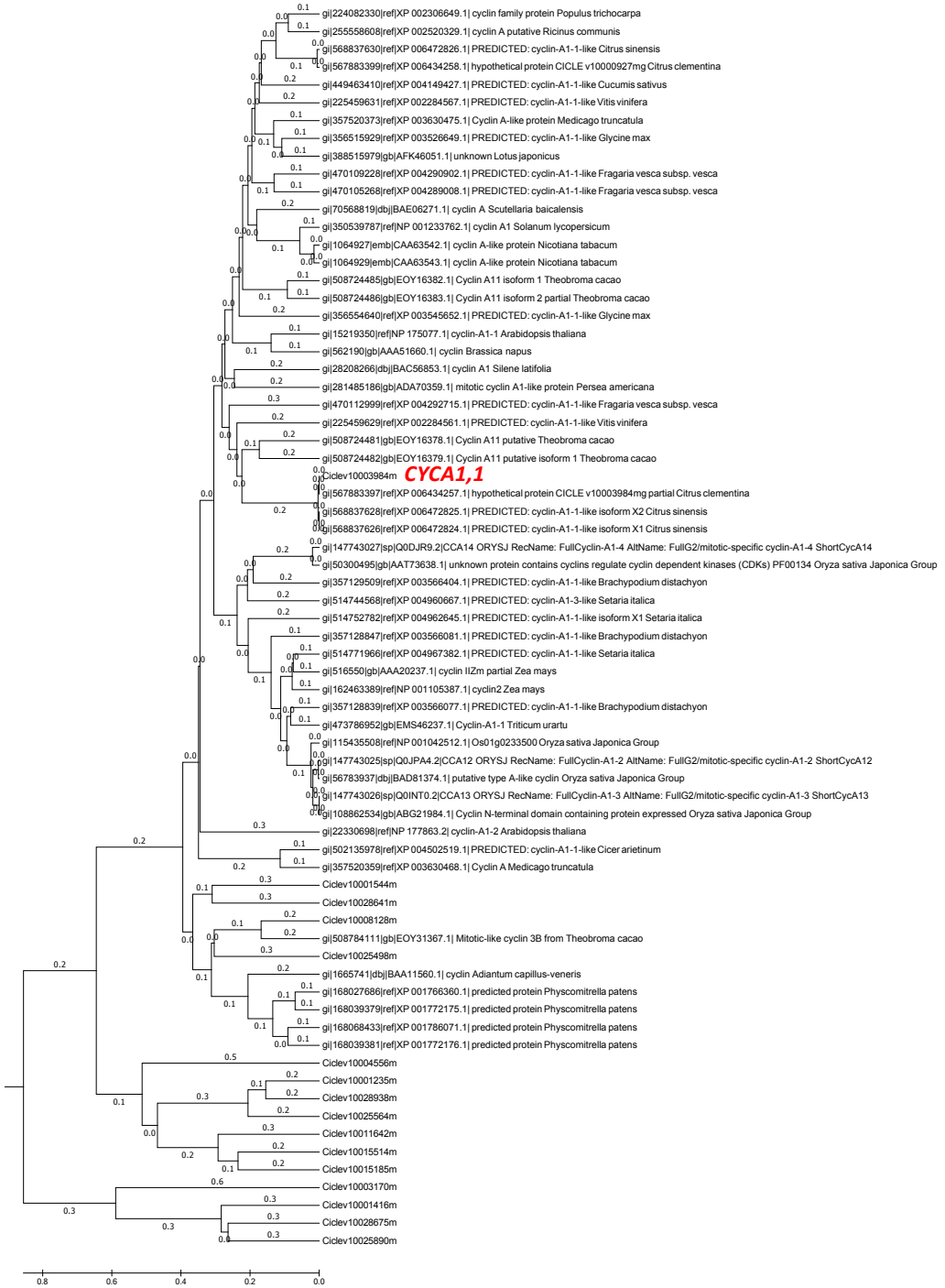


**Figure S4**. Phylogenetic analysis of CYCA1,1 in *C. clementina*. The tree is a detailed magnification of the most specific region of the maximum tree of a larger alignment (see below). One EST was found in the *C. clementina* genome. The EST selected for gene expression studies is Ciclev10003984m, named *CYCA1,1*.



# RESULTS

**Chapter I.**

## 1 GA biosynthesis location during fruit set in seeded and parthenocarpic citrus fruit

In seeded genotypes, several findings firmly support the hypothesis that GA biosynthesis may be specifically produced in the pro-embryo of newly fertilized ovules. However, it is still not clear if this signal originates only in developing seeds, being thereafter relocated to the growing ovary, or instead, if GAs are also synthesized in the pericarp after ovule fertilization. On the other hand, GAs may be synthesized in the pericarp of the parthenocarpic fruitlet or otherwise relocated from other parts of the tree (i.e. leaves). To establish the specific site of GA biosynthesis, measurements were recorded taken for GA concentration and *GA20ox2* and *GA3ox1* expression and *in situ* localization in the ovules and pericarp tissues of 'Navel Barnfield' (sterile and parthenocarpic) and 'Pineapple' (self-compatible and seeded) sweet orange cultivars.

### *1.1 Flowering, fruit set and seed set*

The mean number of flowers per tree was significantly higher in 'Navel Barnfield' than in 'Pineapple' trees (16.780 and 10.153, respectively; $P<0.05$, Fig. 5). On the contrary, fruit set percentage in 'Navel Barnfield' was significantly lower than in 'Pineapple' (4.9 and 7.2 %, respectively; $P<0.05$, Fig. 5) at the end of the physiological fruit drop. 'Navel Barnfield' and 'Pineapple' trees presented 521±24 and 853±49 fruits, respectively (Fig. 6). As expected, no seeded fruit was found in 'Navel Barnfield' trees whereas 'Pineapple' fruits had 14.3 seeds on average (Fig. 6).

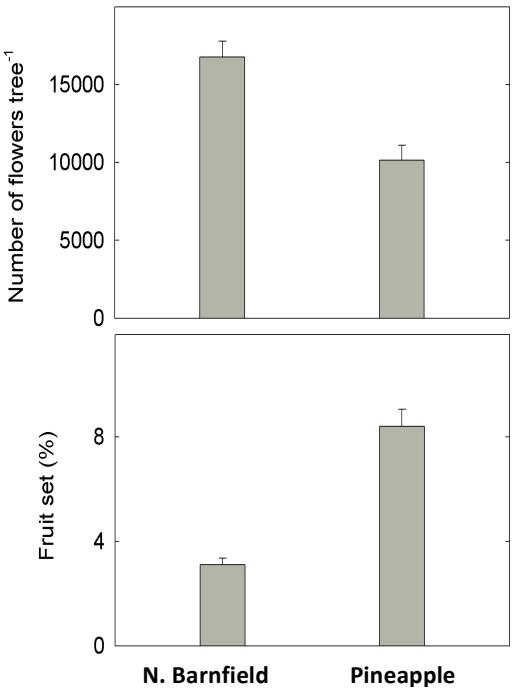



**Figure 5**. Number of flowers per tree and final fruit set (%) of *C. sinensis* cvs. 'Navel Barnfield' (seedless) and 'Pineapple' (seeded). Data are means ± ES of 15 trees per cultivar.Differences are significant (P<0.05).

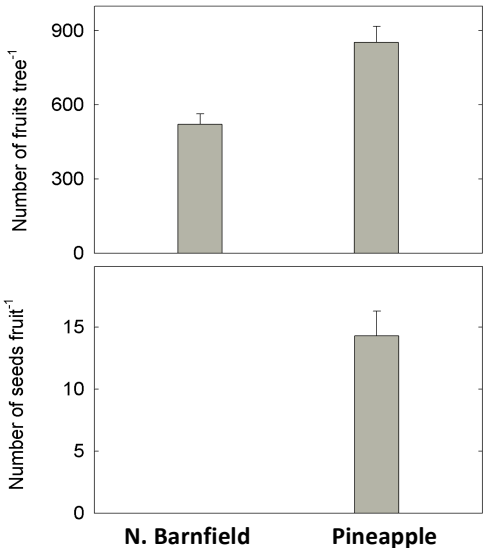



**Figure 6**. Number of fruits harvested per tree and number of seeds per fruit in *C. sinensis* cvs. 'Navel Barnfield' (seedless) and 'Pineapple' (seeded). Data are means ± ES of 15 trees per cultivar and 25 fruits per cultivar for harvest seediness evaluation, respectively. Differences are significant (P<0.05).

To determine the specific phenological growth stage at which the 'Pineapple' ovules were fertilized, pollen tube growth along the style and the ovary was evaluated at 5 phenological growth stages (Fig. 7).


**Figure 7**. Phenological growth stages of *C. sinensis* cv. Pineapple flowers 1: preanthesis (growth stage 59 on the BBCH scale); 2: anthesis (growth stage 63 on the BBCH scale); 3: petal fall I (growth stage 67 on the BBCH scale); 4: petal fall II (growth stage 69 on the BBCH scale); 5: fruitlet (growth stage 71 on the BBCH scale).

Pollen tubes grew intercellularly between the parenchyma cells of the stigmatoid tissue, reaching the style at growth stage 69 on the BBCH scale (stage 4, Fig. 7). Almost 90% of the style was traversed by the pollen tubes by this stage, and also the first pollen tubes could be observed at the end of the stylar canals, in the ovary, reaching the micropyle of the ovules (Fig. 8). Thus, growth stage 69 on the BBCH scale was selected for the study of the specific site of GA biosynthesis given that 'Pineapple' ovules were fertilized.

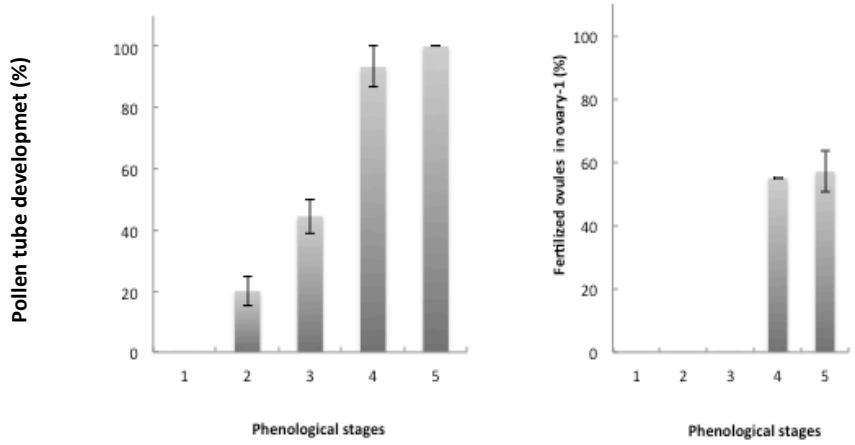



**Figure 8**. Time-course of pollen tube development and ovule fertilization in the *C. sinensis* cv. 'Pineapple' sweet orange. Phenological stages were 1: preanthesis (growth stage 59 on the BBCH scale); 2: anthesis (growth stage 63 on the BBCH scale); 3: petal fall I (growth stage 67 on the BBCH scale); 4: petal fall II (growth stage 69 on the BBCH scale); 5: fruitlet (growth stage 71 on the BBCH scale). Data are means ± ES of 10 flowers per date.

To this end, ovaries were dissected in two parts: i) ovary walls, i.e. the outer pericarp containing the mesocarp and the exocarp, and ii) the locular content (also called *segment*) which at the evaluated stage contains the emerging juice sacs and the fertilized and sterile ovules in 'Pineapple' and 'Navel Barnfield' oranges, respectively (Fig. 9). No differences were observed among ovules from both cultivars, which presented an intact nucella covered by two coats and attached to the ovary wall by the funiculus (Fig 9E and F). Primordia of juice sacs appeared just before the flower opened in both cultivars and took place on the adaxial surface of each carpel. No significant differences were found in the number of juice sacs per ovary (103±3 in 'Pineapple', 120±8 in 'Navel Barnfield'). Regarding the pericarp, the thin-walled parenchyma cells that underlay the epidermis of fruitlets were compactly arranged and presented an intense cell division (data not shown). No significant differences were found among cultivars in the pericarp anatomical parameters observed (Fig 9).

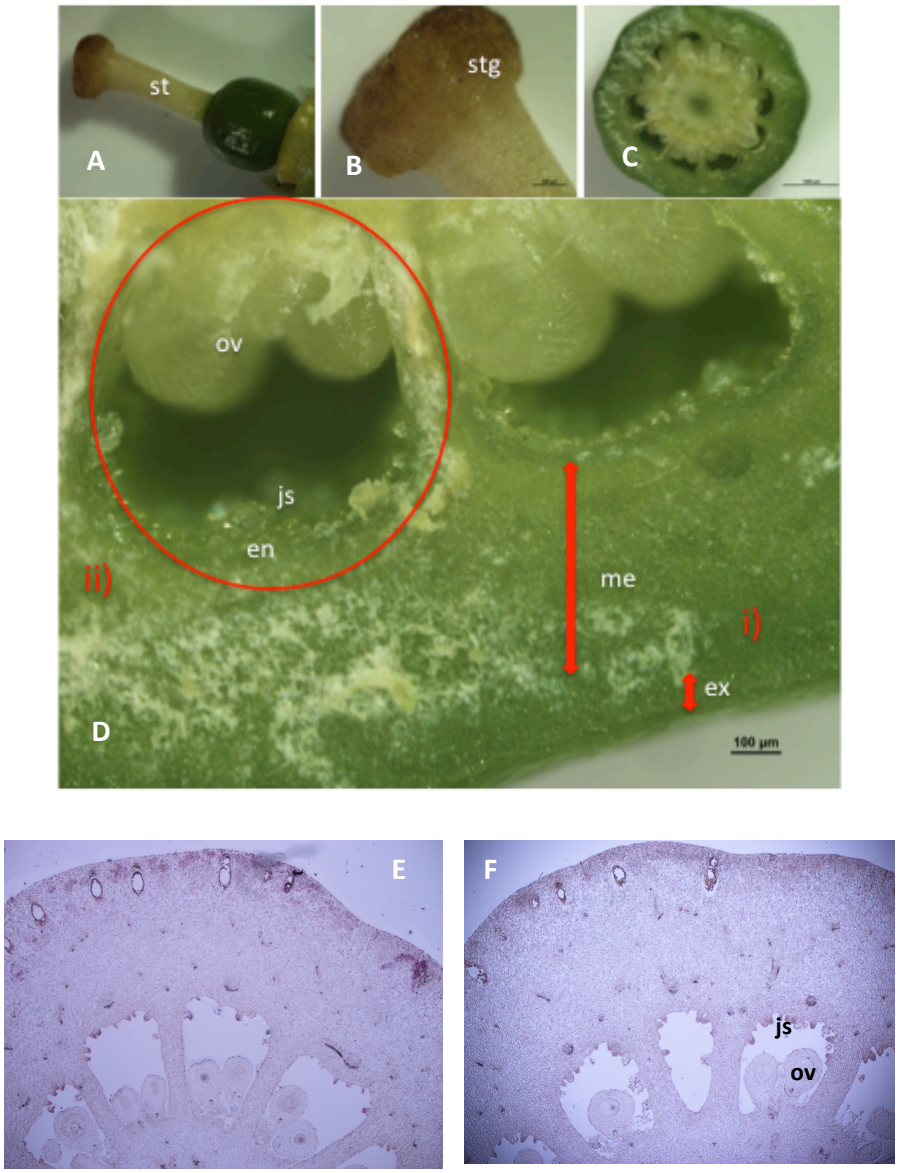



**Figure 9**. Anatomical and histological characteristics of the 'Pineapple' (A-E) and 'Navel Barnfield' (F) sweet orange flowers at the growth stage 69 of the BBCH scale. In D: i (arrows; ex+me) and ii (circle; ov+en+js) indicate the sets of tissues separated for hormone analysis; Stigma (stg), style (st), ovules (ov), juice sacs (js), endocarp (en), exocarp (ex), mesocarp (me).

### *1.2 Gibberellin concentration in ovules and pericarp*

The results of the GA identification analyses showed no qualitative differences between cultivars. Both the 13-hydroxylation and the non-hydroxylation pathways were identified and, in general, 'Pineapple' orange had higher endogenous contents than 'Navel Barnfield'. The gibberellins of the 13-hydroxylation pathway ($GA_{19}$, $GA_{20}$ and $GA_1$) presented higher values and more pronounced differences between varieties than the GAs of the non-hydroxylation pathway ($GA_{24}$, $GA_9$ and $GA_4$) (Figs. 10 and 11). Thus, the concentration of $GA_{19}$ was found to be the highest, followed by $GA_{29}$ (the inactive product of $GA_{20}$), $GA_{20}$, and the bioactive $GA_1$ (Fig. 10).

The quantification of GAs in the ovules+endocarp (ov+en) and ovary walls of 'Pineapple' and 'Navel Barnfield' oranges revealed significant differences. The GA content in the ov+en was always significantly higher than in the ovary walls, regardless of the cultivar (Figs. 10 and 11). The concentration of bioactive gibberellins, $GA_1$ and $GA_4$, in the ov+en was more than 2-fold higher than in the ovary walls of both cultivars, being the differences greater in the case of 'Pineapple' ovary. The catabolic products of $GA_{20}$, $GA_1$ and $GA_9$, i.e. $GA_{29}$, $GA_8$ and $GA_{51}$ respectively, showed minor or even no differences between cultivars but were always higher in the ov+en compared to ovary walls regardless of the cultivar (Figs. 10 and 11). It is interesting to note that the ratios $GA_{20}/GA_{29}$, $GA_1/GA_8$, and $GA_9/GA_{51}$ were significantly higher in the 'Pineapple' ov+en (0.22, 0.93 and 4.4) than in the 'Navel' ov+en (0.11, 0.5 and 1.5) suggesting higher catabolic activity (*GA2ox* activity) in the 'Navel' ovary.

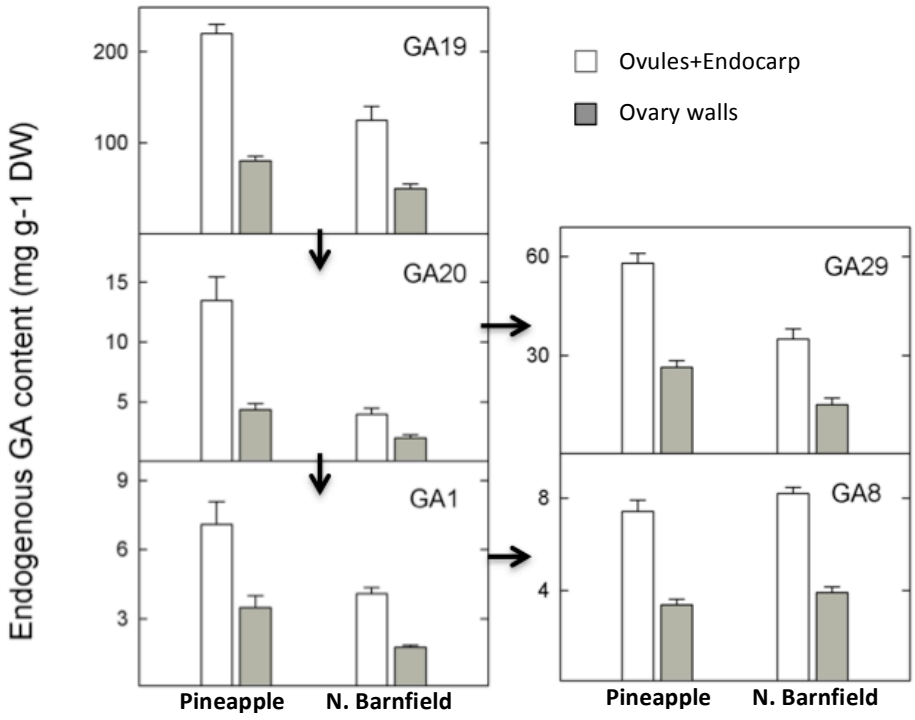



**Figure 10**. Endogenous gibberellin content of early-13-hydroxylation pathway, $GA_{19}$, $GA_{20}$, $GA_1$, and catabolites $GA_{29}$, $GA_8$, in the *C. sinensis* cvs. 'Navel Barnfield' (seedless) and 'Pineapple' (seeded) at stage 69 on the BBCH growth scale. GAs were measured in the ovules + endocarp (white bars) and in the ovary walls (exocarp + mesocarp; grey bars). Data are means ± ES of 3 sets of 30 flowers per cultivar. All differences between tissues are significant ($P<0.05$).

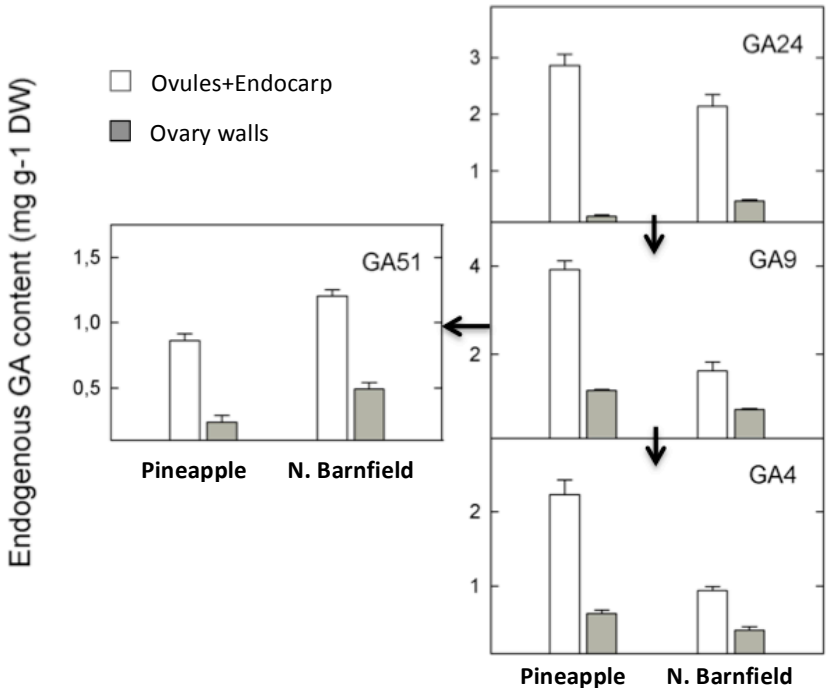



**Figure 11**. Endogenous gibberellin content of non-13-hydroxylation pathway, $GA_{24}$, $GA_9$, $GA_4$, and catabolite $GA_{51}$, in the *C. sinensis* cvs. 'Navel Barnfield' (seedless) and 'Pineapple' (seeded) at the stage 69 on the BBCH growth scale. GAs were measured in the ovules + endocarp (white bars) and in the ovary walls (exocarp + mesocarp; grey bars). Data are means ± ES of 3 sets of 30 flowers per cultivar. All differences between tissues are significant ($P<0.05$).

### *1.3 GA20ox2 and GA3ox1 gene expression*

*GA20ox2* gene expression in ovaries was strongly affected by the cultivar and the analysed tissue (Fig. 12). The relative expression of the *GA20ox2* gene in the ov+en was significantly higher than that determined in the ovary walls of both cultivars, 'Pineapple' ov+en showing significantly higher *GA20ox2* relative expression than 'Navel Barnfield' ov+en (2- to 6-fold, Fig. 11). By contrast, *GA20ox2* relative expression did not significantly differ between cultivars when studied in the ovary walls (Fig. 12).

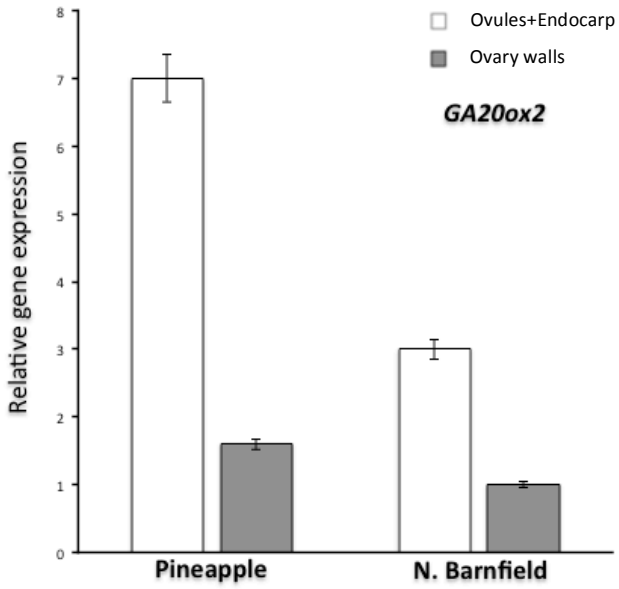



**Figure 12**. The *GA20ox2* relative expression in the *C. sinensis* cvs. 'Pineapple' (seeded) and 'Navel Barnfield' (seedless) at stage 69 of the BBCH growth scale. The gene expression was measured in the ovules + endocarp (white bars) and in the ovary walls (exocarp + mesocarp; grey bars). Data are means ± ES of 3 qRT-PCR replicates. Expression levels were calculated relative to the lowest value ('N. Barnfield' ovary walls). Differences between tissues are significant ($P<0.05$).

Maximum relative transcript abundance of *GA3ox1* gene was found in 'Pineapple' in the ov+en being 1.6 greater than that in 'Navel Barnfield' ov+en (Fig. 13). At this developmental stage, *GA3ox1* activity was higher in ovules than in ovary walls of both cultivars, and *GA3ox1* relative expression did not significantly differ between cultivars when studied in the ovary walls (Fig. 13).

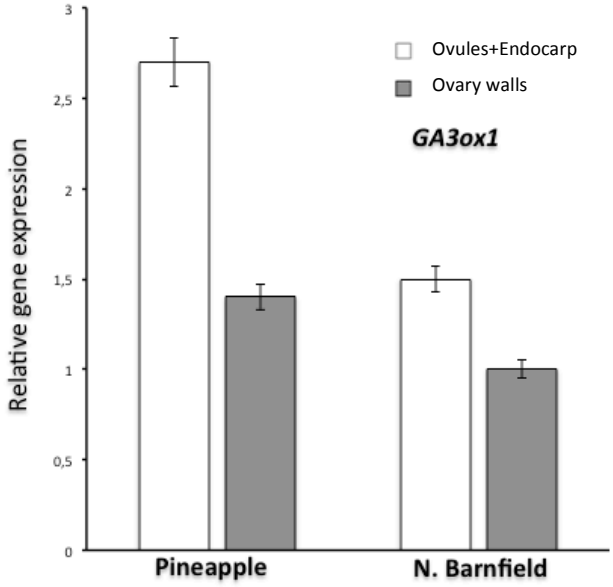



**Figure 13**. The *GA3ox1* relative expression in the *C. sinensis* cvs. 'Pineapple' (seeded) and 'Navel Barnfield' (seedless) at stage 69 of the BBCH growth scale. The gene expression was measured in the ovules + endocarp (white bars) and in the ovary walls (exocarp + mesocarp; grey bars). Data are means ± ES of 3 qRT-PCR replicates. Expression levels were calculated relative to the lowest value (N. Barnfield ovary walls). Differences between tissues are significant ($P<0.05$).

**Chapter II**

## 2. Gibberellins promote fruit growth triggering ovary cell division at the onset of parthenocarpic Citrus fruit development

### *2.1 Cell division and parthenocarpic ovary growth*

Satsuma cv. Owari and Clementine cv. Clemenules ovary growth differed significantly and particularly on the most advanced dates of the experiment. In Satsuma mandarin, parthenocarpic ovary growth could be observed some 5 d before anthesis and was clearly apparent at anthesis (Fig. 14A). Two days after anthesis, the ovary growth was triggered and the ovary grew according to an exponential pattern during the following 15 d, resulting in a 7-fold increase in weight (Fig. 14A). On the other hand, Clementine ovary growth was nil at pre-anthesis and significantly low after anthesis, only resulting in a 2-fold increase in weight 15 days after anthesis (DAA) (Fig. 14A). As a result of this difference in the parthenocarpic ability Satsuma fruit set was significantly higher (96%) than that of Clementine fruit (68%) ($P<0.05$) (Fig. 14E-14F).

Ovary growth was mainly due to pericarp cell division, and fruit weight significantly correlated the number of cell layers in the pericarp ($y = 2.0 x - 34.9$; $R^2 = 0.978$). In Satsuma mandarin, the cell division rate in the pericarp was triggered just after anthesis, as it occurred for fruit growth rate, and the number of cell layers in the pericarp increased 2.7-fold from anthesis to 15 d later (Fig. 14B). On the other hand, the number of cell layers in the Clementine mandarin pericarp was almost constant during the period studied, and only a slight increase was observed 10 DAA (Fig. 14B).

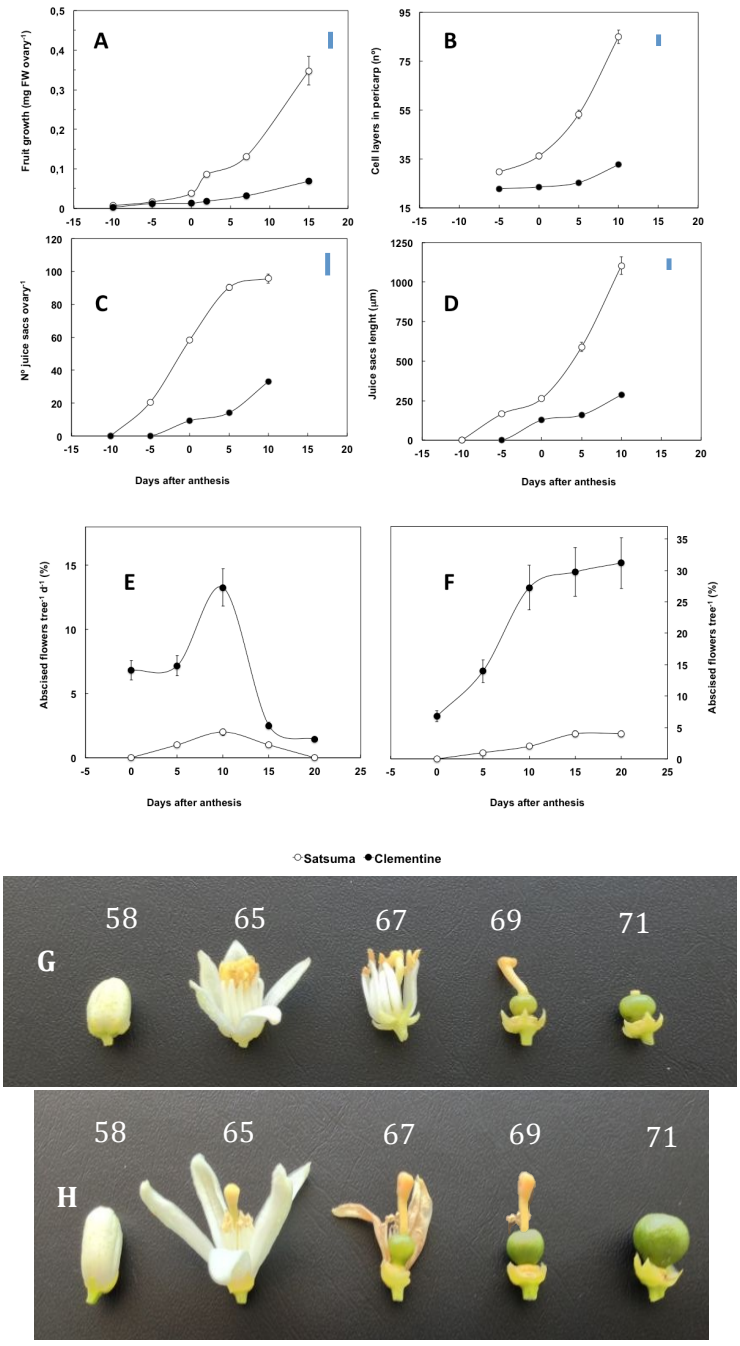



**Figure 14.** Ovary development of Satsuma mandarin (white circles) and Clementine mandarin (black circles) at the onset of fruiting (A-F). Phenological growth stages of the Clementine (G) and Satsuma ovaries (H). Data are means ± ES. (A) N=50 ovaries. (B-D) N= 5 ovaries. (E-F) N= 15 trees. Blue bar indicates LSD (P<0.01).

Interestingly, the Satsuma endocarp showed active meristematic activity before anthesis, when clear protuberances appeared in the locule wall, which continued to develop into the juice vesicles (Fig. 15). The number of juice vesicles in the Satsuma ovary increased 4.5-fold at 10 DAA (Fig. 14C; Fig. 15), and their length also increased significantly from anthesis (250 µm vesicle$^{-1}$) to 10 days later (1200 µm vesicle$^{-1}$) (Fig. 14D; Fig. 15). On the other hand, Clementine locule walls were very few and these free of protuberances at preanthesis (Fig. 15) while short juice vesicles were observed from anthesis onwards (Fig. 14C and D).

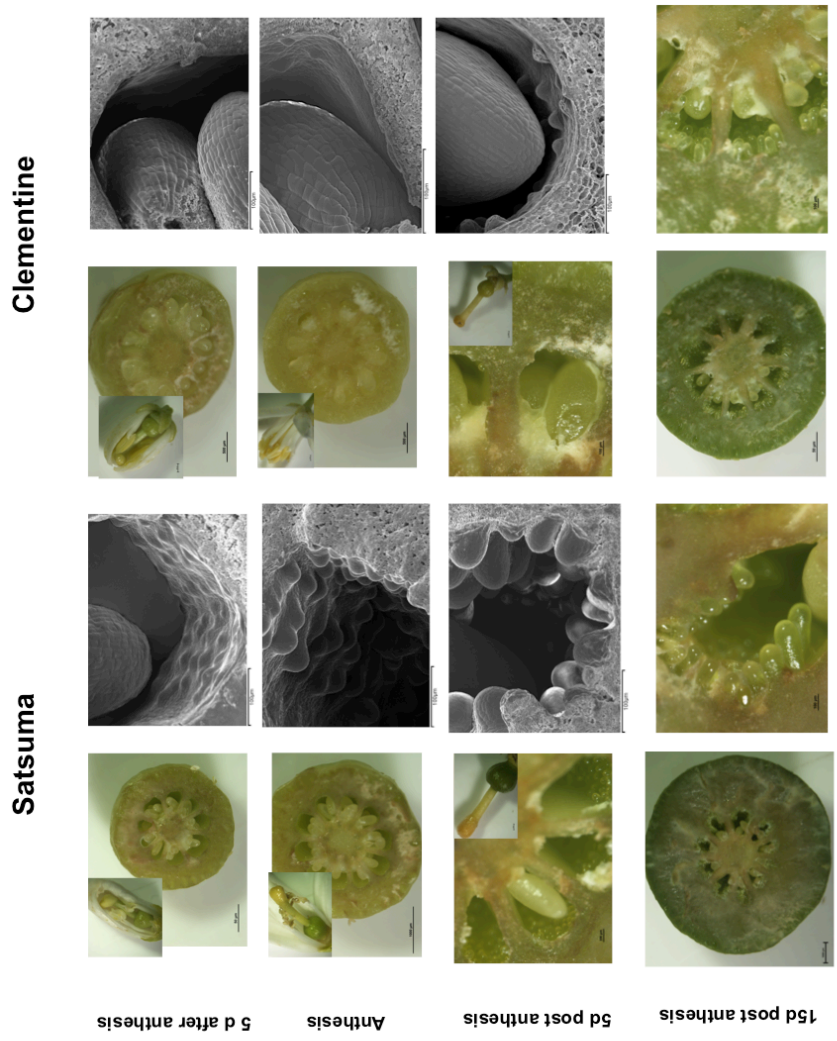



**Figure 15.** Time-course of juice sac growth from the carpel walls (endocarp) of Satsuma ovary (left) and Clementine ovary (right).

### *2.2 Constitutive activation of cell division and GA biosynthesis*

In the Satsuma ovary, expression of the specific cyclin *CycA1,1* gene from the G2 stage of the cell cycle was downregulated 1.3-fold at anthesis compared to 10 days earlier (Fig. 16). Interestingly, *CycA1,1* gene expression was quickly recovered 5 DAA reaching the highest relative expression 10 DAA (2.1-fold) and paralleling the cell division rate observed in both the pericarp, i.e. number of cell layers (Fig. 14B), and the juice vesicles (Fig. 14D). On the other hand, the Clementine ovary showed a strong downregulation of the *CycA1,1* gene expression (3.8-fold) from -10 DAA to anthesis (Fig. 16). Furthermore, *CycA1,1* gene expression was not recovered in the Clementine ovary after anthesis, like it did in the Satsuma ovary, and the former always showed lower relative expression than in the pre-anthesis stage , 10 days before anthesis (DBA) (Fig. 16). Results are in accordance with the rate of cell division found in the pericarp and endocarp, reinforced by the significant correlation between *CyCA1,1* expression pattern and the number of cell lines in the pericarp ($y = 27.9 x + 14.0$; $R^2 = 0.87$), the number of juice vesicles in the ovary ($y = 48.9 x - 2.9$; $R^2 = 0.88$), and the longitude of the juice vesicles ($y = 460 x - 69$; $R^2 = 0.85$).

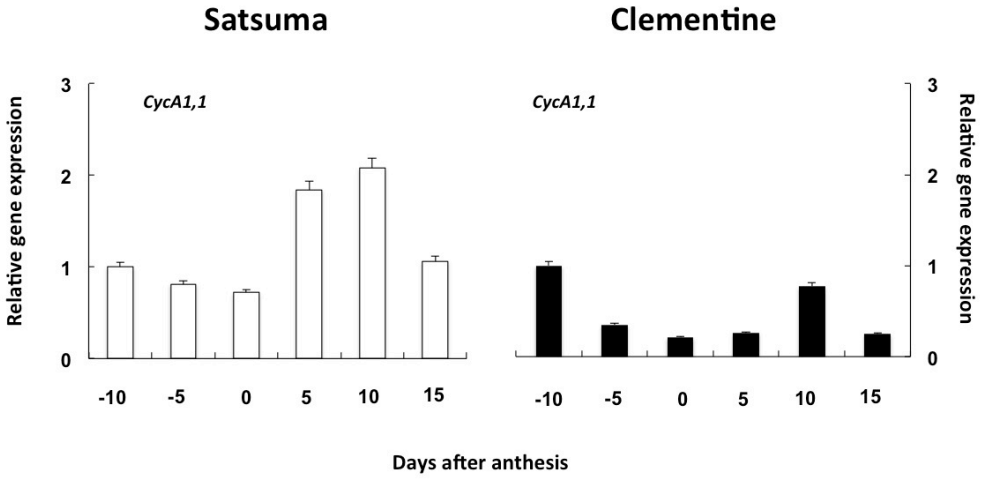



**Figure 16**. *CycA1,1* expression in Satsuma mandarin (white bars) and Clementine mandarin (black bars) ovaries during the onset of fruiting. Data are means ± ES of of 3 qRT-PCR replicates.

Satsuma and Clementine ovaries differed clearly in the expression pattern of the genes involved in GA metabolism. Satsuma ovary showed constitutive activation of the growth-active GA biosynthesis during the pre-anthesis stage. Specifically, *GA20ox2* was transiently upregulated from -10 DBA to anthesis reaching up to 5-fold relative expression increases. *GA3ox2* expression was significantly higher (19-fold) at -5 DBA and constitutively expressed until 5 DAA while *GA3ox1* varied less (Fig. 17). On the other hand, Clemenules ovary showed the opposite activity, that is, downregulation of *GA20ox2* and *GA3ox2* expression from -10 DAA to anthesis. *GA3ox1* also varied little (Fig. 17).

Growth-active GAs biosynthesis was strongly upregulated 10 and 15 DAA in both Satsuma and Clementine ovaries. It is worth noting that (1) the highest increase in GA3ox activity was produced 5 days earlier in the Satsuma ovary (10 DAA) compared to the Clementine ovary (15 DAA); (2) the GA3ox expression was strongly upregulated in both species to a similar level (400-fold); (3) upregulation was produced for different genes, *GA3ox2* and *GA3ox1* for Satsuma and Clementine ovaries, respectively (Fig. 17).

Expression of the GA deactivation genes was alternatively upregulated and downregulated in both Clementine and Satsuma within different time frames (Fig. 18). In general, *GA2ox1* expression was downregulated from anthesis onwards reaching a lower level in the Satsuma ovary compared to the Clementine. On the other hand, *GA2ox2*, *GA2ox3* and *GA2ox4* expression was upregulated to a maximum achieved 5-10 DAA. The highest expression level was that of *GA2ox3* in the Clementine ovary followed by *GA2ox2* in the Satsuma ovary (Fig. 18).

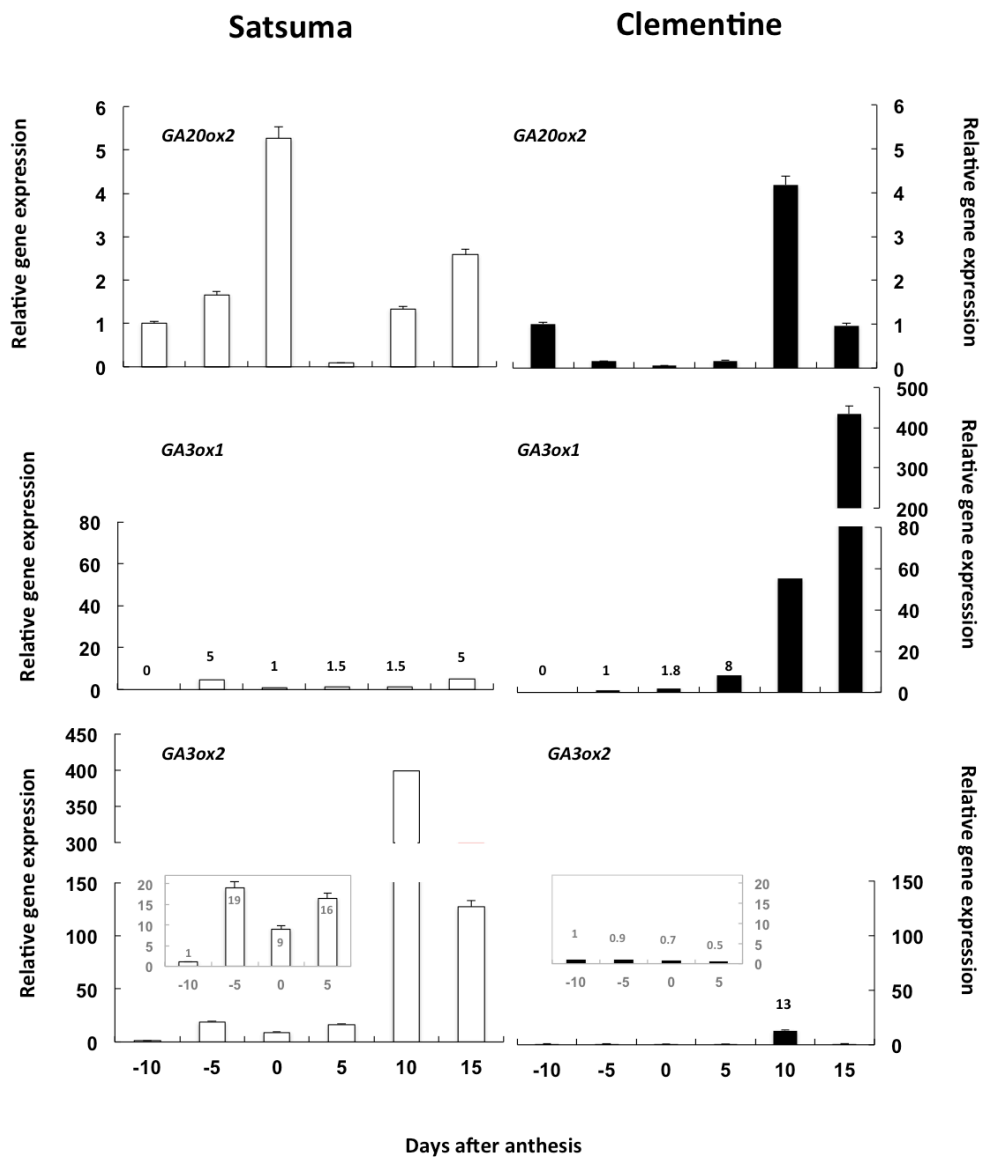



**Figure 17**. *GA20ox2*, *GA3ox1* and *GA3ox2* expression in Satsuma mandarin (white bars) and Clementine mandarin (black bars) ovaries during the onset of fruiting. Data are means ± ES of 3 qRT-PCR replicates.

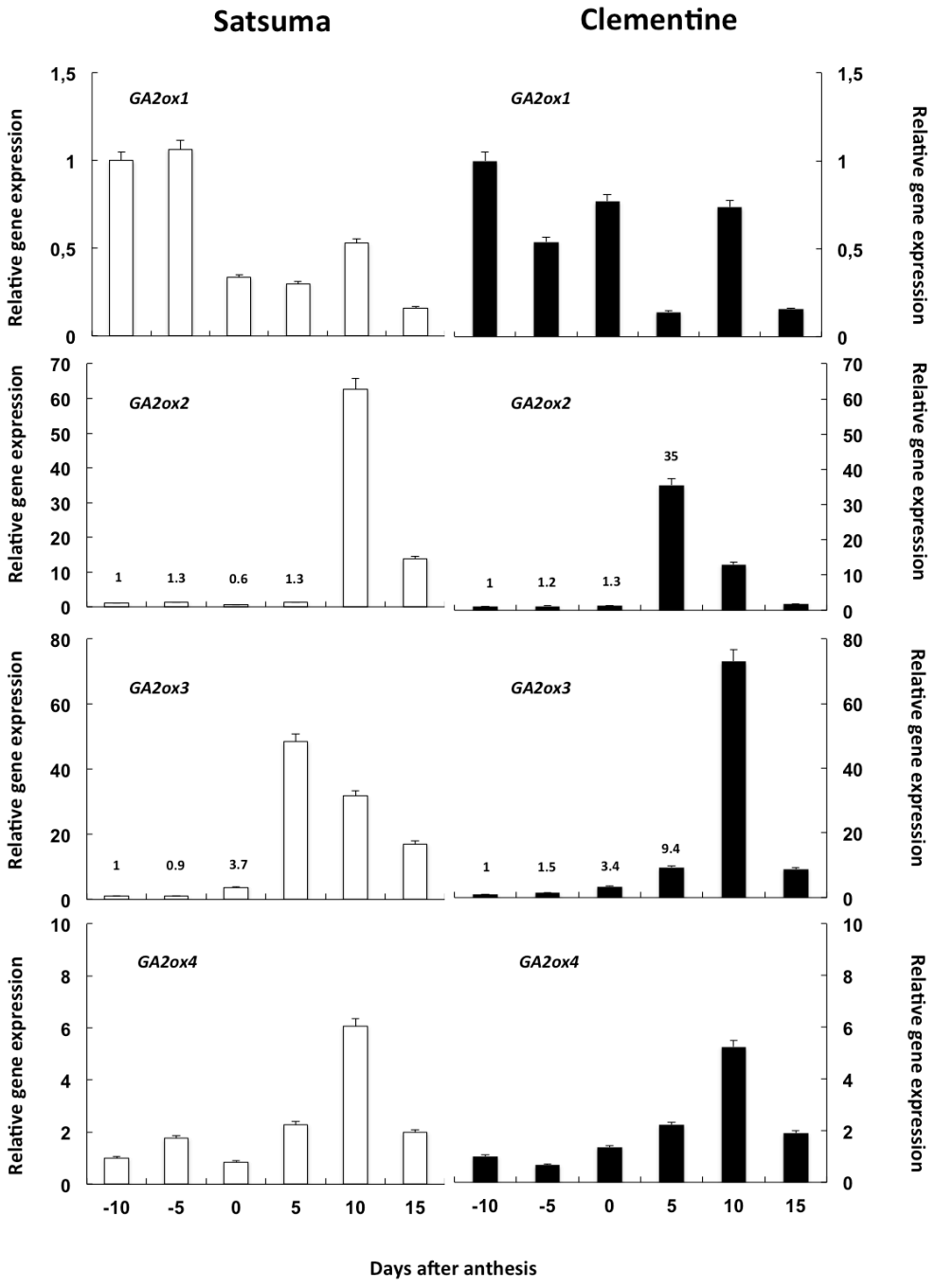



**Figure 18**. *GA2ox-1,2,3,4* expression in Satsuma mandarin (white bars) and Clementine mandarin (black bars) ovaries during the onset of fruiting. Data are means ± ES of 3 qRT-PCR replicates.

### *2.3 Localization of GA20ox2 transcripts by in situ hybridization*

Satsuma and Clementine ovaries showed clear differences in the expression of *GA20ox2* gene at anthesis at the tissue level. In agreement with RT-PCR data, the *in situ* hybridization analysis of the *GA20ox2* transcripts revealed a weak expression of the gene in the Clementine ovary at anthesis (Fig. 19A, C) while a strong hybridization signal was observed in the Satsuma ovary at the same stage (Fig.19B, D). In the Clementine ovary, transcripts were specifically observed in the outermost tissue layers of the pericarp, i.e. the exocarp, and in the marginal vascular bundles (Fig. 19A, C). On the other hand, the Satsuma ovary showed *GA20ox2* transcripts in the whole pericarp (exo-, meso-, and endocarp), in the juice vesicles, in the septa, in the central axis, and in the vascular bundles (Fig. 19B, D). No signal was found in ovules of either Satsuma or Clementine ovaries (Fig. 19). Thirty days after anthesis in the Satsuma growing fruitlet, *GA20ox2* transcripts were specifically located in the endocarp and juice sacs, and with lower intensity in the exocarp; no signal was found in the mesocarp (Fig. 19F-G). This mesocarp-to-endocarp *GA20ox2*-expression transition was also observed in the cell division rate. At anthesis and 10 days later, cell division was mainly observed in the mesocarp whereas at 30 DAA cell division was above all restricted to the endocarp and growing juice sacs whereas no cell division was observed in the mesocarp (Fig. 19H).

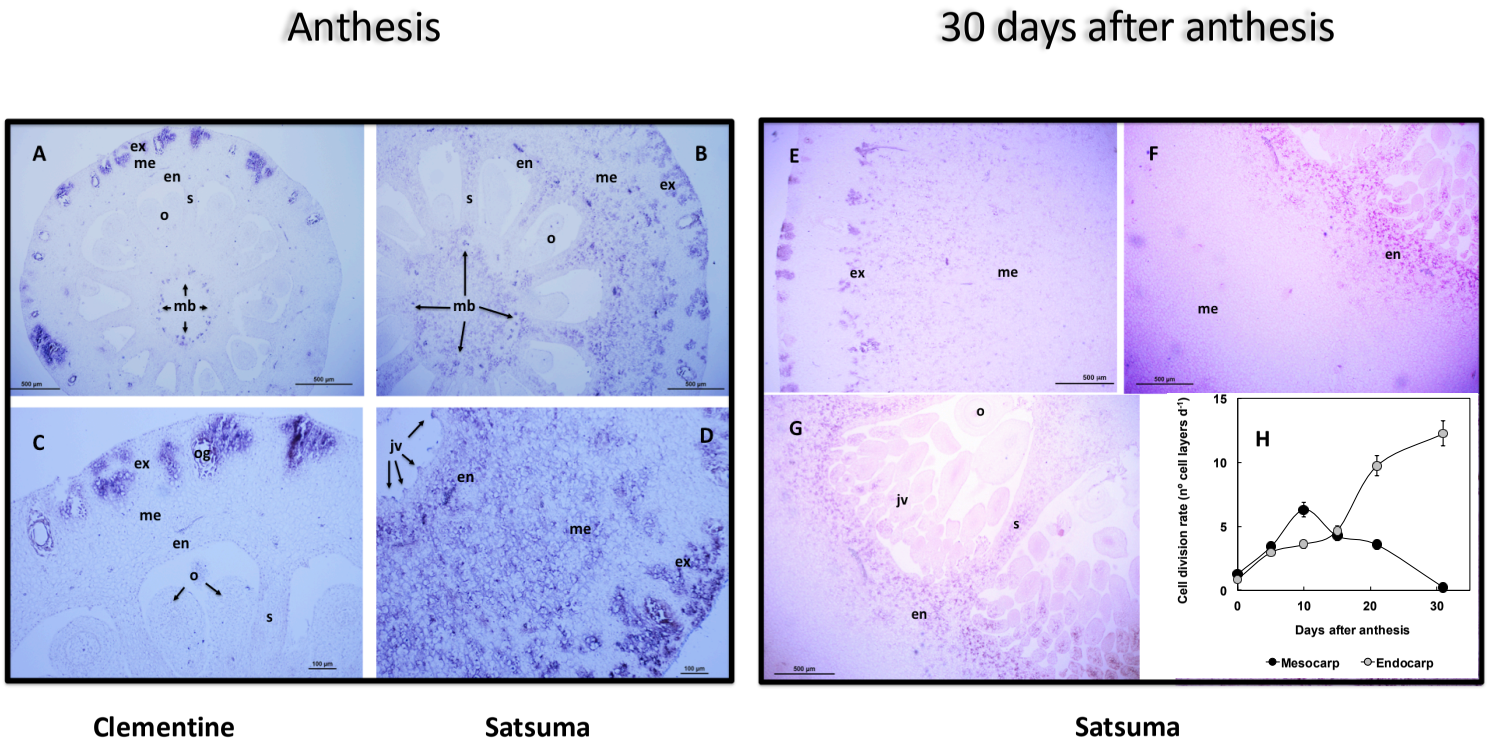



**Figure 19**. *In situ hybridization* of GA20ox2 transcripts in the Clementine (low parthenocarpic, A,C) and Satsuma (high parthenocarpic, B, D, E, F, G, H) ovaries at anthesis (A-D) and 30 days later (E-G). H: Cell division rate in Satsuma mesocarp and endocarp; en: endocarp; ex: exocarp; jv: juice vesicles; mb: marginal bundles; me: mesocarp; o: ovules; s: septa.

### *2.4 Hormonal regulation of parthenocarpy*

From anthesis to 31 days later Satsuma and Clementine fruitlets increased in weight from 0.038 to 2.12 g ovary$^{-1}$, i.e. 56-fold, and from 0.013 to 0.167 g ovary$^{-1}$, i.e. 13-fold, respectively (Fig. 20). Exogenous applications of $GA_3$ promoted parthenocarpic fruit development in the Clementine mandarin, measured as the increase in fruit weight; thus, the treatment significantly increased fruit weight 21 and 31 DAA compared to the control. On the other hand, $GA_3$ did not stimulate Satsuma mandarin fruit weight (Fig. 20). The application of PBZ produced a significant reduction in fruit weight very a few days before the treatment in both species. Thus, PBZ-treated fruits showed reduced weight compared to untreated fruits from 5 d after the treatment onwards.

Treatment of Clementine ovary with $GA_3$ significantly promoted cell division in all the tissues, exo-, meso- and endocarp, significantly increasing the number of cell layers in the pericarp, the number of juice vesicles and its length (Fig. 20). Interestingly, the effect of $GA_3$ was first observed in the endocarp where meristematic activity was significantly boosted 7 DAA and, therefore, the number of juice vesicles arising from the locule wall was significantly higher in the treated ovary compared to the control (Fig. 20). In Satsuma ovaries, the $GA_3$ treatment boosted cell division temporally, increasing the number of cell layers in the pericarp and the number of juice sacs per ovary only 7 DAA (Fig. 20). On the other hand, PBZ produced a significant reduction in cell division rate a very few days before the treatment (i.e. 7 DAA) in both the ovary walls and the juice vesicles in both varieties (Fig. 20).

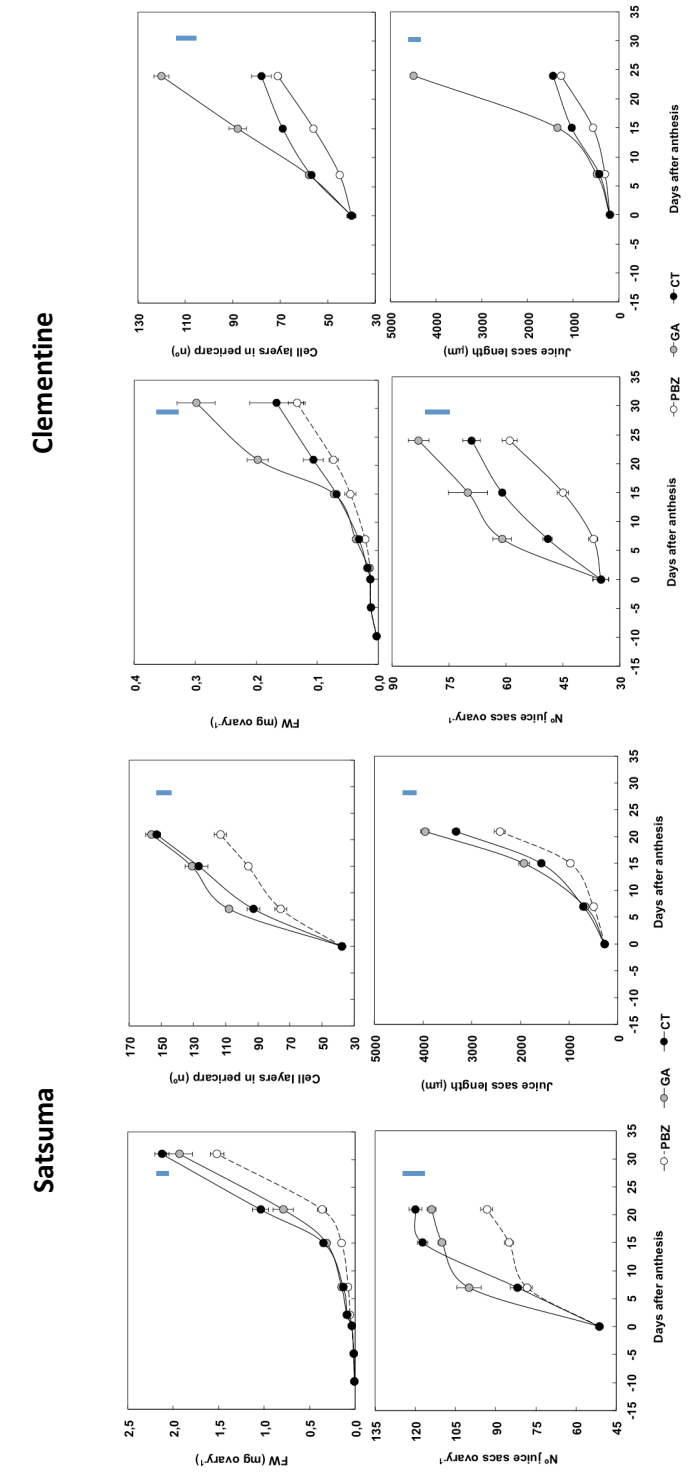



**Figure 20**. Effect of $GA_3$ (10 mg $l^{-1}$) and PBZ (1000 mg $l^{-1}$) on cell division and parthenocarpic fruit development of Satsuma and Clementine mandarins. Data are means ± SE of 10 fruits per treatment and date. 150 fruits from 4 trees per cultivar were used in the experiment. Treatments were applied directly to the fruitlet at anthesis. Control (CT) fruits were not treated. Blue bar indicates LSD (P<0.01).

In agreement with the histological study, $GA_3$ stimulated *CycA1,1* relative expression whereas PBZ inhibited it in both Satsuma and Clementine ovaries, although differing in the temporal pattern of expression (Fig. 21). Compared to the control, treatment of Satsuma ovaries with $GA_3$ produced a 2.4-fold increase in *CycA1,1* gene expression 6h after the treatment, the effect being lost 48h after the treatment (Fig. 21). On the other hand, $GA_3$ up-regulation of *CycA1,1* gene expression in the Clementine ovary occurred 7 DAA, after an initial depressive effect 6h after treatment (Fig. 21). PBZ treatment induced *CycA1,1* downregulation in both Satsuma and Clementine mandarins, the effect being more pronounced in the latter (Fig. 21).

Treatments also modified GA biosynthesis genes expression in both Satsuma and Clementine ovaries resulting in significant changes in GAs ovary concentration. In particular, the application of $GA_3$ modified the 13-hydroxilation pathway, increasing $GA_{20}$ and $GA_1$ and decreasing the precursors $GA_{53}$ and $GA_{19}$, whereas PBZ significantly modified both, the 13-hydroxilation and the non-hydroxilation pathways, in accordance with its inhibitory effect of the oxidation of *ent*-kaurene to *ent*- kaurenoic acid (Fig. 22). Treatment with $GA_3$ induced *GA20ox2* downregulation, the effect being particularly evident in Satsuma, whereas *GA3ox* activity was upregulated in both species although within different time frames and for different genes (Fig. 23). Interestingly, *GA3ox* activity paralleled that of *CycA1,1* in response to the $GA_3$ treatment in both Satsuma and Clementine ovaries (Fig. 21, Fig. 23). Finally, the *GA2ox* genes also showed induction by $GA_3$ treatment. On the other hand, Satsuma and Clementine GA-metabolism genes revealed a similarly answer to the PBZ treatment: *GA20ox* activity was dowregulated while *GA3ox* upregulated 6h after the treatment, these differences being lost 48h after the treatment. Attending to the effect on bioactive GAs, $GA_1$ and $GA_4$, Satsuma mandarin $GA_1$ concentration paralleled *CYCA1,1* gene expression in response to $GA_3$ producing a 5-fold temporarily increase (Fig 21, Fig. 22). Compared to the control, PBZ did not reduce $GA_1$ but $GA_4$ until 7d after the treatment (Fig. 22). In the Clementine mandarin, $GA_3$ significantly increased $GA_1$ concentration (4-fold) whereas PBZ did not modify it compared to the control, but significantly reduced $GA_4$ from 6h to 7d after the treatment (Fig. 22).

Satsuma untreated ovaries showed higher $GA_1$ concentration (almost 4-fold higher) than Clementine ovaries, as expected (Fig. 4).

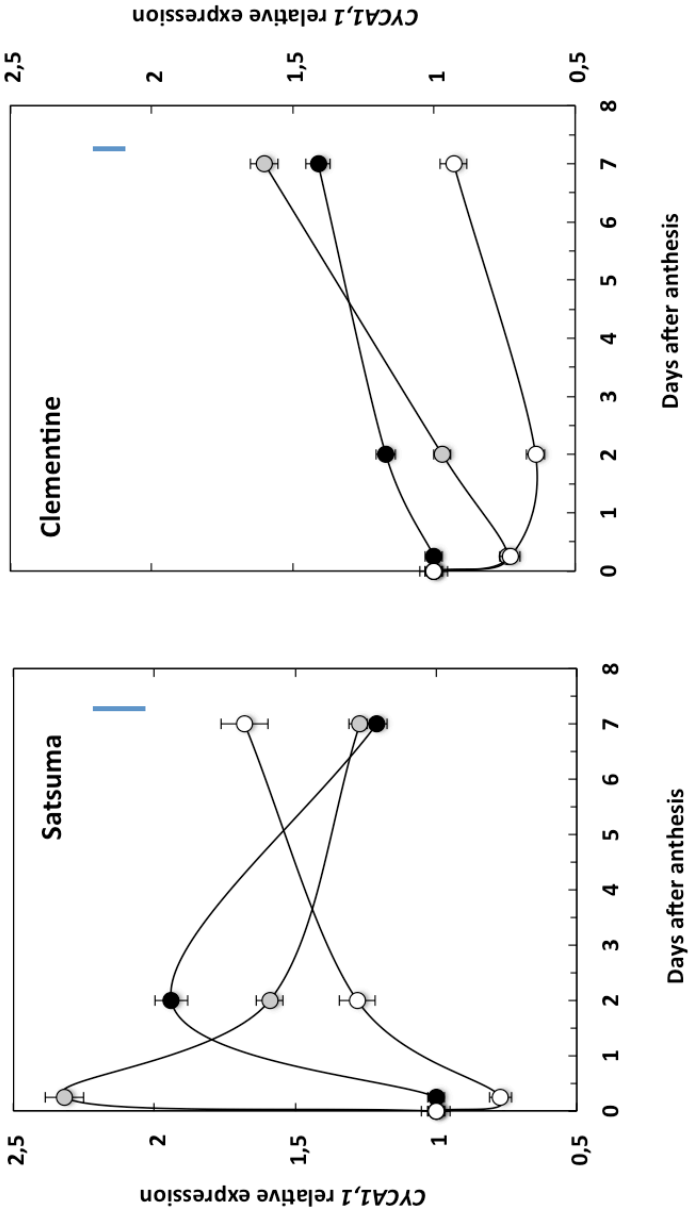



**Figure 21**. Effect of $GA_3$ (10 mg $l^{-1}$) and PBZ (1000 mg $l^{-1}$) on *CycA1,1* gene expression of Satsuma and Clementine mandarins. Data are mean ± SE of 3 qRT-PCR experiments. A total of 150 fruits from 4 trees per cultivar were used in the experiment. Treatments were applied directly to the fruitlet at anthesis. Control (CT) fruits were not treated. Blue bar indicates LSD ($P<0.01$).

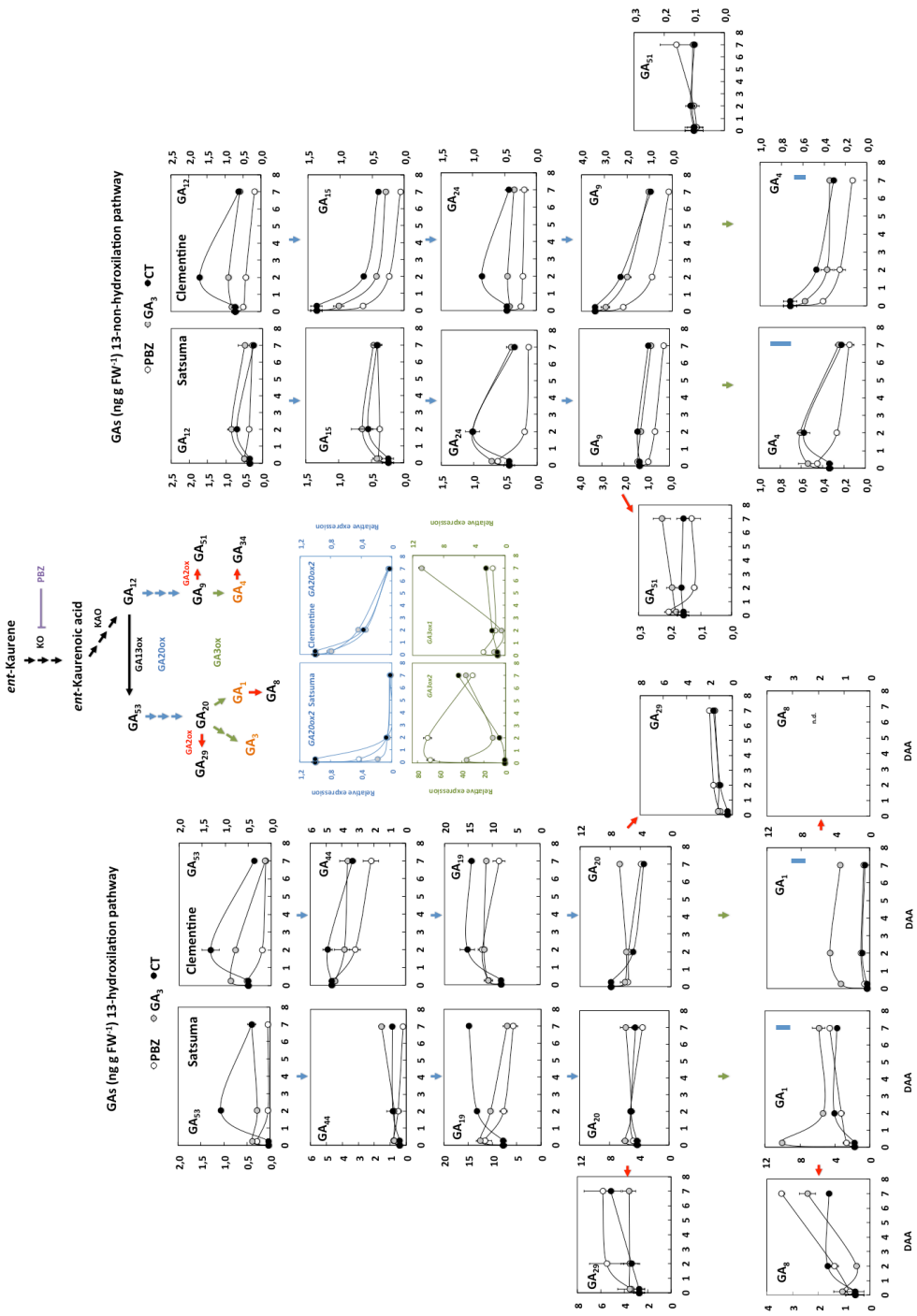



**Figure 22**. Effect of $GA_3$ (10 mg $l^{-1}$) and PBZ (1000 mg $l^{-1}$) on *GAs* ovary concentration of Satsuma and Clementine mandarins. Data are mean ± SE of 3 biological replicates. A total of 150 fruits from 4 trees per cultivar were used in the experiment. Treatments were applied directly to the fruitlet at anthesis. Control (CT) fruits were not treated. Blue bar indicates LSD ($P<0.05$).

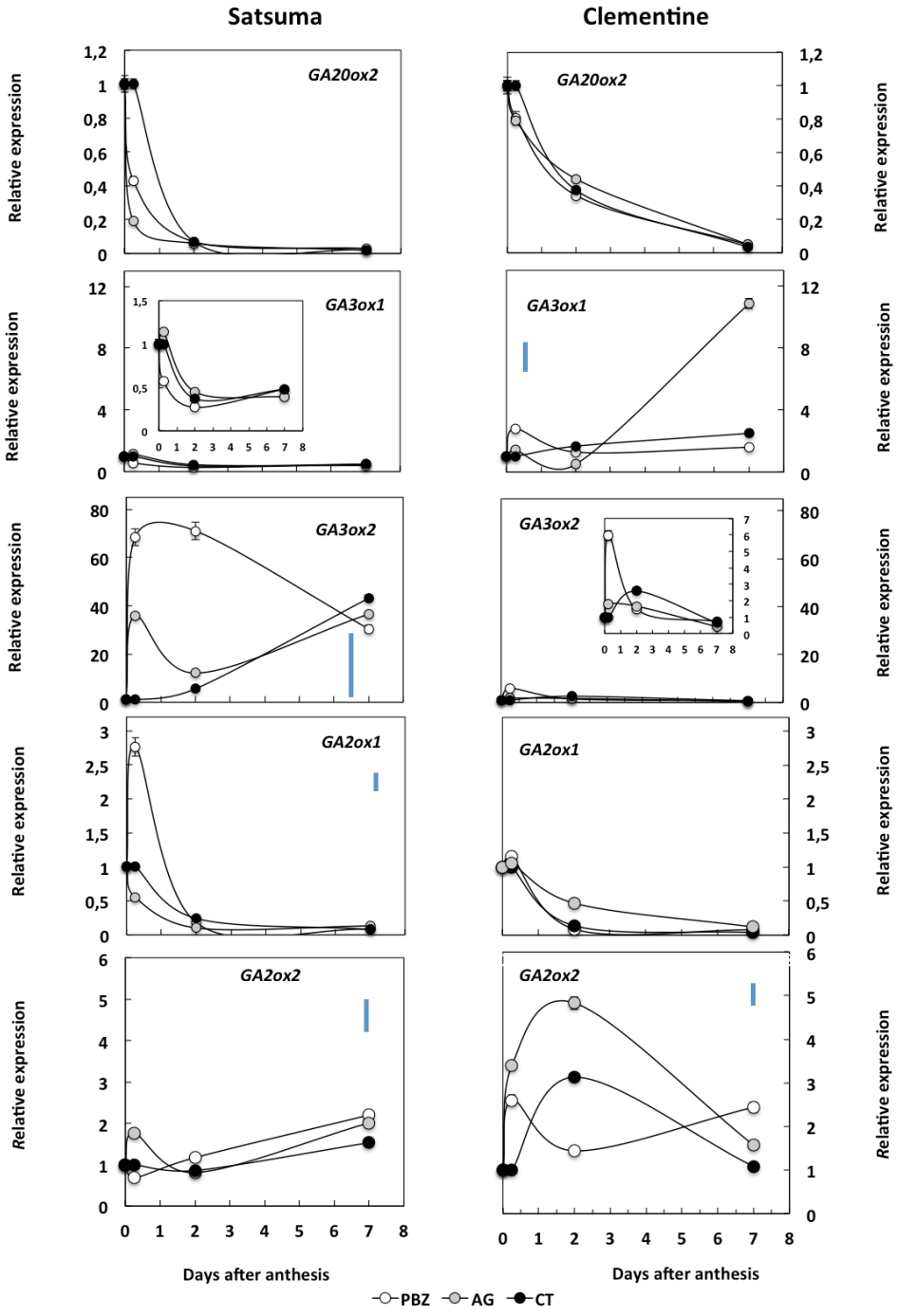



**Figure 23**. Effect of $GA_3$ (10 mg $l^{-1}$) and PBZ (1000 mg $l^{-1}$) on *GAox* gene expression of Satsuma and Clementine mandarins. Data are mean ± SE of of 3 qRT-PCR experiments. A total of 150 fruits from 4 trees per cultivar were used in the experiment. Treatments were applied directly to the fruitlet at anthesis. Control (CT) fruits were not treated. Blue bar indicates LSD (P<0.05).

## Chapter III

## 3. Self-pollination and parthenocarpic ability in developing ovaries of self-incompatible Clementine mandarins (*Citrus clementina*)

In this chapter, the effect of self-pollination on fruit set and active gibberellin ($GA_1$ and $GA_4$) content in the ovary was studied in 'Marisol' and 'Clemenules' Clementine mandarins. These mandarins show high and low tendency to develop parthenocarpic fruits, respectively. Time-course for carbohydrates, active gibberellins, auxin (IAA) and abscisic acid (ABA) content in the ovary were also analysed. Unlike un-pollinated control flowers, self-pollination did not stimulate fruit set and GA content in the ovary, in any of the cultivars studied.

### *3.1 Pollen grain germination, pollen tube growth and ovule abortion*

Three days after pollination (DAP) pollen germination was significantly higher in 'Clemenules' (19%) than in 'Marisol' flowers (8%) (Table 1). Three days later (6 DAP) pollen germination did not increase significantly (data not shown). As expected, no pollen grains were found in un-pollinated flowers (Table 1).

After germination, pollen tubes grew between the parenchyma cells of the stigmatoid tissue reaching the stylar canals. A self-incompatibility reaction was triggered in the style of both cultivars. Pollen tubes presented altered morphology with heavy callose deposits at the tip, and they impeded growth in the style 9 DAP. Only 9 and 13% of the style was traversed by the longest pollen tube in 'Marisol' and 'Clemenules' flower, respectively (Table 1). No pollen tubes were observed at the base of the style or the ovary.

The absence of fertilization resulted in natural ovule abortion. Ovules from both 'Marisol' and 'Clemenules' un-pollinated and self-pollinated flowers did not show symptoms of abortion until 12 DAP when 4 – 6% of the ovules per ovary had a callose deposit at the chalazal end (Table 1). Thereafter, the pattern of ovule abortion was similar for both cultivars in self-pollinated flowers and un-pollinated flowers with no significant differences for a given cultivar, but the differences between the two

cultivars did become significant for both types of flowers (Table 1). Specifically, at 18 and 21 DAP, the percentage of aborted ovules was significantly higher in 'Marisol' un-pollinated ovaries (57 and 71%, respectively) than in 'Clemenules' un-pollinated ovaries (42 and 50%, respectively). Similar percentages were found for self- pollinated flowers: 59 and 69% for 'Marisol', and 48 and 56% for 'Clemenules' (Table 1). As expected, all mature fruits were seedless for self-pollinated and un-pollinated flowers (data not shown).

**Table 1.** Pollen grain germination, pollen tube growth and ovule abortion in Clementine mandarin cvs. Marisol and Clemenules. Pollen germination on the stigma was evaluated by counting 200 pollen grains per flower in 5 flowers per treatment. Pollen tube development in the style was determined as the percentage of the style traversed by the longest pollen tube in each flower. Ovule abortion was evaluated by counting the number of ovules with callose layering at the chalazal end. Different letters in the same line indicate significant differences ($P<0.05$). DAP, days after pollination; UP, un-pollinated; SP, self-pollinated.

| | | Marisol | | Clemenules | |
|---|---|---|---|---|---|
| | DAP | UP | SP | UP | SP |
| Pollen grain germination (%) | 3 | | 8 ± 2 a | | 19 ± 2 b |
| Pollen tube growth (% of style) | 3 | | 0 | | 0 |
| | 6 | | 8 ± 5 | | 8 ± 6 |
| | 9 | | 9 ± 5 | | 13 ± 6 |
| Ovule abortion (%) | 9 | 0 | 0 | 0 | 0 |
| | 12 | 4 ± 2 | 5 ± 2 | 6 ± 3 | 6 ± 2 |
| | 15 | 19 ± 3 | 21 ± 1 | 17 ± 3 | 20 ± 6 |
| | 18 | 57 ± 5 b | 59 ± 4 b | 42 ± 2 a | 48 ± 6 ab |
| | 21 | 71 ± 3 b | 69 ± 4 b | 50 ± 3 a | 56 ± 4 a |

### *3.2 Fruit set, abscission pattern and fruit growth*

The results of these experiments indicate that fruit set was significantly higher in 'Marisol' than in 'Clemenules' mandarin, averaging 850 ± 50 and 180 ± 20 fruits per tree, respectively. The two varieties showed two waves of fruitlet abscission and there were significant differences. The first wave occurred at weeks 2 to 5 after pollination. At this time, 'Marisol' mandarin had a lower fruitlet abscission rate (i.e. percentage of fruitlets abscised a day) and a higher proportion of fruit set 35 DAP (82%) than 'Clemenules' mandarin (64%) (Fig. 24; $P<0.05$). The second wave of abscission took place during weeks 6 to 9 after pollination (Fig. 24), coinciding with the onset of the rapid fruit growth stage (Fig. 25).

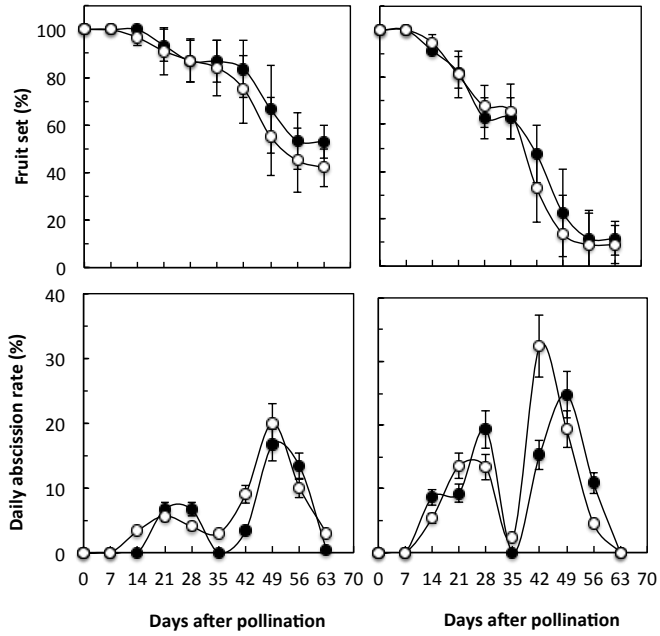



**Figure 24**. Parthenocarpic fruit set and fruit abscission rate for un-pollinated (black circles) and self-pollinated (white circles) flowers from 'Marisol' (left) and 'Clemenules' (right) Clementine mandarin trees. The vertical bars show the SE. Data are means of 5 trees.

In both cultivars, the second fruit abscission wave was markedly more pronounced than the first one, 'Marisol' abscising a lesser proportion of fruit than 'Clemenules' mandarin (Fig. 24). Particularly, 42 and 49 DAP the abscission rate for 'Marisol' un-pollinated ovaries was 5% and 16%, respectively, whereas for 'Clemenules' it reached 15% and 27%, respectively (Fig. 24). As a result, at the end of fruitlet abscission (63 DAP) fruit set in 'Marisol' (47%) differed significantly from that in Clemenules (10%) (Fig. 24; $P<0.05$).

Self-pollination did not enhance fruit set for either cultivar, and the fruit abscission pattern was similar for self-pollinated and un-pollinated ovaries (Fig. 24).

Fresh fruit weight of remaining fruits did not differ significantly between cultivars or treatments in the first wave of abscission (Fig. 25). However, at the onset of the second wave of fruitlet abscission, fruits on 'Marisol' trees had grown significantly more, compared to 'Clemenules' trees, for both pollinated and un-pollinated flowers. Thus, between 26 DAP and 36 DAP, 'Marisol' fruitlet increased 84% in fresh weight, whereas 'Clemenules' fruitlet increased 38%, with no significant differences due to self-pollination (Fig. 25).

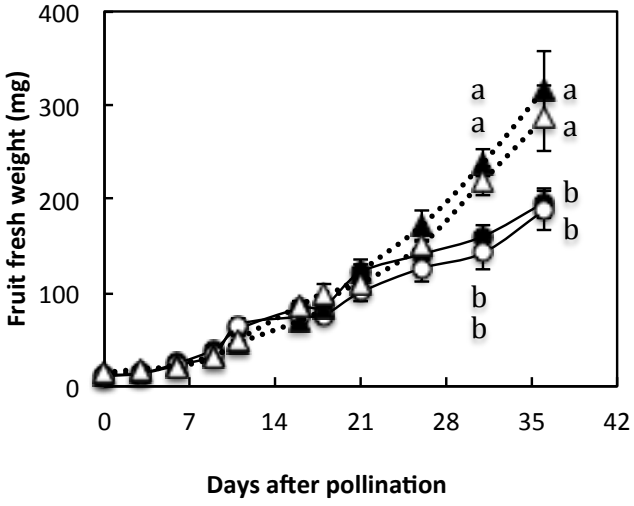
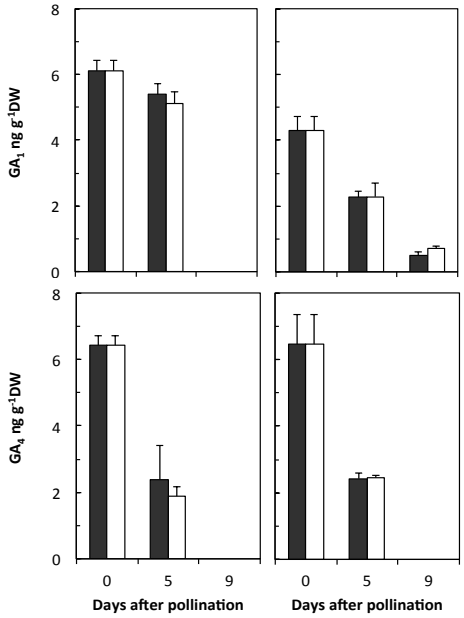



**Figure 25**. Time-course for fresh fruit weight of developing ovaries from un-pollinated (black triangles) and self-pollinated (white triangles) flowers for 'Marisol' (triangles) and 'Clemenules' (circles) Clementine mandarin trees. The error bars show the SE. Data are means of 10 to 100 fruits per treatment because of abscission. Means followed by different letters on the same day are significantly different at $P \leq 0.05$.

### *3.3 $GA_1$ and $GA_4$ levels in developing ovaries*

Endogenous $GA_1$ and $GA_4$ concentrations in the ovaries of 'Marisol' and 'Clemenules' decreased continuously from 0 to 9 DAP ($P < 0.001$). At 0 and 5 DAP, un-pollinated ovaries of 'Marisol' had a significantly higher $GA_1$ content than un-pollinated ovaries of 'Clemenules' ($P < 0.001$) (Fig. 26). The $GA_1$ concentration in 'Marisol' was 1.5-fold that of 'Clemenules' at 0 DAP, and five days later (5 DAP) the difference increased 2.5-fold. However, 9 DAP, $GA_1$ concentration in 'Marisol' ovaries was nil, whereas that of 'Clemenules' ovaries was 0.7 ng $g^{-1}$ DW (Fig. 26). The $GA_4$ concentration in ovaries of un-pollinated flowers in both cultivars was similar at 0 and 5 DAP, and nil four days later (9 DAP) (Fig. 26).

Self-pollination did not modify $GA_1$ or $GA_4$ ovary concentration in either cultivar ($P = 0.94$ and $P = 0.78$, respectively) (Fig. 26).



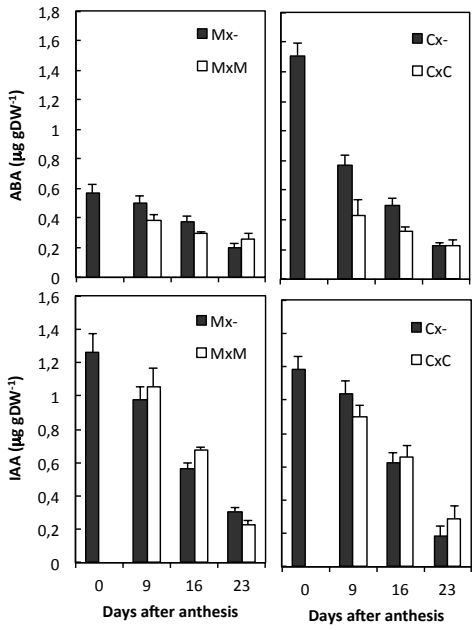

| | *P*-values from ANOVA | | | | | |
|---|---|---|---|---|---|---|
| | A: Cultivar | B: Pollination | C: DAP | AxB | AxC | BxC |
| $GA_1$ | < 0.001 | 0.94 | <0.001 | 0.74 | <0.001 | 0.91 |
| $GA_4$ | 0.68 | 0.78 | <0.001 | 0.73 | 0.78 | 0.78 |

**Figure 26**. $GA_1$ and $GA_4$ content in ovaries of un-pollinated (black bars) and self-pollinated (white bars) flowers from 'Marisol' (left) and 'Clemenules' (right) Clementine mandarin trees. The vertical bars show the SE. DAP: Days after pollination.

### *3.4 ABA and IAA content*

The time-course for ABA and IAA content in the ovary was studied during the first wave of fruit abscission (Fig. 27). At 0 DAP, ABA concentration in the ovary of un-pollinated 'Clemenules' flowers was 2.7 higher than that of un-pollinated 'Marisol' flowers ($P < 0.001$) (Fig. 27). Nine days later the difference was only 1.5 higher for 'Clemenules' ovary and disappeared at 16 DAP (Fig. 27). Self-pollinated flowers had significantly lower concentrations of ABA in the ovary up to 16 DAP (Fig. 27). Differences between ovaries from self-pollinated and un-pollinated flowers were not statistically significant 23 DAP.

The IAA concentration decreased by 70% in the ovary of un-pollinated flowers of both cultivars from 0 to 23 DAP (Fig. 27). The reduction pattern was similar not only for 'Marisol' and 'Clemenules' ovaries, but also between un-pollinated and self-pollinated flowers (Fig. 27). No changes in the IAA concentration in the ovary of either cultivar were attributed to self-pollination.




| | *P*-values from ANOVA | | | | | |
|---|---|---|---|---|---|---|
| | A: Cultivar | B: Pollination | C: DAP | AxB | AxC | BxC |
| ABA | < 0.001 | 0.007 | <0.001 | 0.18 | <0.001 | <0.001 |
| IAA | 0.47 | 0.66 | <0.001 | 0.66 | 0.89 | 0.73 |

**Figure 27.** ABA and IAA contents in un-pollinated (black bars) and self-pollinated (white bars) ovaries of 'Marisol' (left) and 'Clemenules' (right) Clementine mandarin trees. The vertical bars show the SE. DAP: Days after pollination.

### *3.5 Carbohydrate content*

The time-course for carbohydrate content in the ovary was studied during the first wave of fruit abscission (Fig. 28). From 0 to 5 DAP, hexoses concentration (glucose+fructose) accumulated significantly in the 'Marisol' ovary (+25%). Thereafter, a dramatic reduction (-70%) was observed until 16 DAP, remaining almost constant up to day 23. In contrast, the hexoses concentration pattern in the 'Clemenules' ovary showed a continuous reduction from 0 to 23 DAP (-54%) (Fig. 28A).

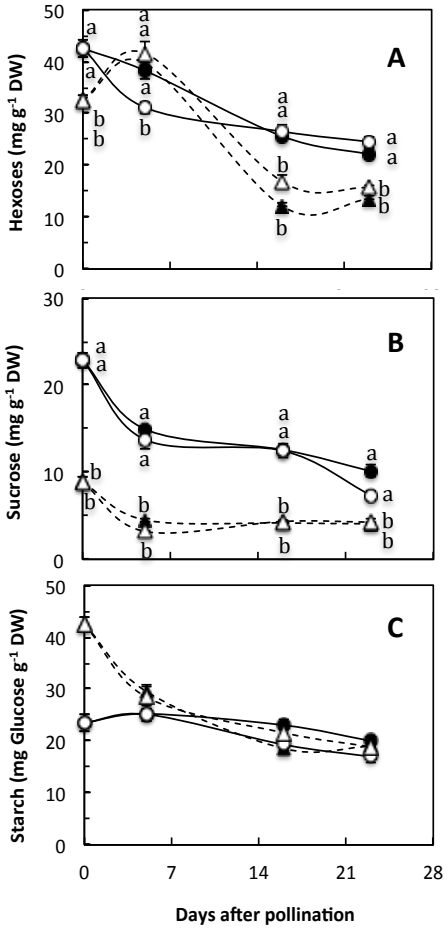



**Figure 28**. The time-course for hexoses (glucose + fructose; **A**), sucrose (**B**) and starch (**C**) concentration in developing ovaries of un-pollinated (black symbols) and self-pollinated flowers (white symbols) from 'Marisol' (triangles) and 'Clemenules' (circles) Clementine mandarin trees. The vertical bars show the SE. Means followed by different letters on the same day are significantly different at $P \leq 0.05$.

Ovary sucrose content decreased in both cultivars from 0 to 23 DAP (Fig. 28B). The reduction pattern was similar for ‘Marisol’ and ‘Clemenules’ ovaries, but the sucrose concentration was consistently and significantly higher in the latter (Fig. 28B). From 0 to 5 DAP and 16 DAP, starch concentration significantly decreased in ‘Marisol’ ovary (- 30% and - 50%, respectively). However, the starch concentration pattern in ‘Clemenules’ ovary did not vary significantly from 0 to 23 DAP (Fig. 28C). Self-pollination did not modify carbohydrate concentrations in the ovaries of either cultivar (Fig. 28).

# DISCUSSION

GA synthesis was analysed in two *C. sinensis* cultivars differing in their seed set ability, in order to determine the specific site of GA synthesis in the ovary of an obligate parthenocarpic cultivar. 'Navel Barnfield' orange is always seedless due to male and female sterility. On the other hand, 'Pineapple' orange is always seedy (14 seeds $fruit^{-1}$ in these experimental conditions) due to cleistogamic self-pollination before anthesis, and was used for comparison.

Ovaries contained both GAs from the 13-hydroxylation pathway leading to $GA_1$, and non-13-hydroxy-GAs at lower levels, leading to $GA_4$, as previously reported (Talón *et al.,* 1990; Ben-Cheikh *et al.,* 1997). GA synthesis was found in the ovules + endocarp (growing juice sacs) and the ovary walls (exocarp+mesocarp) regardless of the cultivars ability to set seeds. This result differs to that found in other species (i.e. *Arabidopsis* or tomato), in which GAs are specifically synthesized in the newly fertilized ovules and not in the pericarp (Ollimpieri *et al.,* 2007; Serrani *et al.,* 2008; Dorcey *et al.,* 2009). In *Arabidopsis*, the GAs synthesized in the fertilized ovules are transported to the pericarp to promote GA signalling and thus coordinate seed and ovary growth (Dorcey *et al.,* 2009).

GA content and synthesis differed significantly between tissues and cultivars, being significantly higher (more than 2-fold) in the ovules+endocarp than in the ovary walls in both cultivars, and higher in the seeded than in the parthenocarpic ovary. The differences in GA content between cultivars correlated the percentage of fruit set as was previously reported (Talón *et al.,* 1990; Ben-Cheikh *et al*., 1997), which was 2.7-fold higher in 'Pineapple' with respect to 'Navel Barnfield'. But the most notable result was that these differences in GAs between cultivars were mainly found in the ovules+endocarp rather than in the ovary walls. The relative expression of *GA20ox2* and *GA3ox1* genes in the ovary walls did not differ significantly between the two cultivars as well as $GA_{19}$, $GA_{20}$, $GA_8$, $GA_{24}$, $GA_9$ and $GA_4$ concentrations. At this early stage of development the increase in thickness of the ovary is mainly the result of cell division in the mesocarp. We did not observed significant differences in pericarp size between the two cultivars, which may explain the latter result.

On the other hand, the different GA content found in the ovules+endocarp between the seeded and seedless cultivars may be due to ovule fertilization since no

significant differences were found in the juice sacs growth between the two cultivars. However, whether the GAs found in the ovules+endocarp of each cultivar is due to the contribution of the endocarp, the ovules or both cannot be precisely determined based on these results. In seeded species, *GAox* biosynthetic genes are upregulated in fertilized not in unfertilized ovules (Dorcey *et al.,* 2009), and we observed a strong *in situ* hybridization of the *GA20ox2* transcripts in the endocarp and growing juice sacs. Accordingly, in the 'Pineapple' orange the fertilized ovules, the endocarp and the growing juice sacs are a source of GAs at this early stage. In the 'Navel Barnfield' parthenocarpic ovary, although fertilization was not produced due to ovule sterility, a constitutive GA biosynthesis in the unfertilized ovules cannot be discarded at this early stage, as it was shown in the parthernocarpic *Pat-2* tomato (Ollimpieri *et al.,* 2007). The physiological degeneration of the unfertilized ovule has been studied in citrus (Tadeo and Primo-Millo, 1998; Mesejo *et al.,* 2007). One of the first indications that an ovule will abort is the synthesis of callose at the chalazal end (Mesejo *et al.,* 2006; 2007). Fifteen days after anthesis the nucellus in 'Navelate' sweet orange shows the first ultrastructural symptoms of degeneration, which is completed several weeks later (Tadeo and Primo-Millo, 1998), and 90% of the 'Owari' Satsuma and 'Clemenules' ovules show callose at the chalazal end 16 and 21 days after anthesis, respectively, revealing that the reduction in the number of ovules is a progressive process depending on the species (Mesejo *et al.,* 2007). Ovule senescence is triggered by ethylene, and a possible mechanism that links the ethylene modulation of the ovule senescence and the $GA_3$-induced fruit set response was proposed (Carbonell-Bejerano *et al.,* 2011). In the 'Navel Barnfield' ovary, $GA_1$ and $GA_4$ synthesized in the ovules+endocarp was also found to be higher than two-fold compared to that in the ovary walls. These results suggest that, apart from the whole pericarp, the unfertilized ovules might very well function as a powerful sink in the parthenocarpic ability of the complete ovary, whereas the newly-fertilized ovule must be the main sink in the pollinated ovary, similar to other species (i.e. pea, Rodrigo *et al.,* 1997).

The quantitative difference in the $GA_1$ and $GA_4$ contents from the two varieties may also have a metabolic explanation. Actually, the 'Pineapple' orange showed significantly higher *GA20ox2* and *GA3ox1* gene expression. Regarding the $GA_1/GA_8$, $GA_{20}/GA_{29}$ and $GA_9/GA_{51}$ ratios, these were significantly higher in 'Pineapple' than in

'Navel' ovary (0.22, 0.93, 4.4, and 0.11, 0.5, 1.5, respectively) suggesting higher catabolic activity (*GA2ox* activity) in the latter.

Overall, in the parthenocarpic cultivars, GA synthesis in the ovary at the fruit set stage occurs not only in the ovary walls but also in the ovules, the endocarp and growing juice sacs. These observations permit hypothesize that, from anthesis to parthenocarpic fruit set, GA accumulation in the ovary walls (exocarp and mesocarp) and in the endocarp may regulate cell division that in turn will lead to ovary and juice sacs growth and, therefore, fruit set.

The autonomous increase in $GA_1$ ovary content during anthesis was suggested as being the stimulus responsible for the process of the parthenocarpic transition from ovary to developing fruitlet in *Citrus*, regardless of the species (Talón *et al.,* 1990, 1992). The results from this Thesis (Chapter II) are in accordance with this suggestion and, further, explain the molecular events related with the specific role of GA during parthenocarpic fruit set, revealing that: (i) GA biosynthetic genes are up-regulated before anthesis in Satsuma ovaries (high parthenocarpic ability) coinciding with the onset of cell division and ovary growth, whereas they are down-regulated in the Clemenules ovaries (low parthenocarpic ability); (ii) GA20ox-2 transcripts are specifically located in the tissues where cell division occurs either at anthesis or 30d later; (iii) the exogenous application of $GA_3$ enhanced cell division and growth whereas PBZ impaired it.

*Constitutive activation of cell division, GA synthesis, and parthenocarpy occurs before anthesis*

Parthenocarpic ovary growth due to the constitutive activation of cell division could be observed some 5 d before anthesis and was clearly apparent at anthesis, particularly in the Satsuma ovary. The expression of the specific cyclin *CycA1,1* gene from the G2 stage of the cell cycle significantly correlated with the rate of cell division found in the pericarp. Similar results were obtained in the parthenocarpic tomato mutant AS-IAA9 particularly for *CycA3* and *Cycb* genes (Wang *et al.,* 2009). The availability of cyclin/cyclin-dependent kinase (CYC/CDK) complexes from the G2/M phase of the cell cycle was reported to mainly restrict cell proliferation during the cell

division phase of fruit development (Malladi and Johnson, 2011), which is in accordance with our results.

As a result of this constitutive activation of cell division and increased ovary growth rate the Satsuma mandarin ranked 96% fruit set (which occurred 15-20 DAA). On the other hand, the low-parthenocarpic Clemenules mandarin showed very low cell division rates and ovary growth the days around anthesis and, therefore, significantly lower fruit set was achieved due to a 30% flower abscission 15 DAA. Moreover, scarce fruitlet development was also recorded in this mandarin 15 DAA onwards, as previously reported (Talón *et al.,* 1992; Zacarias *et al.,* 1995). The strict inverse relationship between ovary growth rate and flower/fruit drop was previously shown (Agustí *et al.,* 1982) and has been explained in terms of competition for carbohydrates (Mehouachi *et al*., 1995; Rivas *et al*., 2006), and this, together with the ability to produce GAs, may modulate the synthesis of ABA, ACC and ethylene, the effector of abscission (Zacarias *et al.,* 1995; Gómez-Cadenas *et al.,* 2000; Iglesias *et al.,* 2006). Particularly, this abscission observed shortly after anthesis appears to be mainly coupled to changes in GA content (Talón *et al.,* 1990; 1992; Zacarías *et al*., 1995; Ben-Cheikh *et al*., 1997).

Satsuma and Clemenules ovaries showed significant differences in the constitutive activation of GA synthesis that determined the flower to fruit transition. The GA biosynthetic genes, *GA20ox* and *GA3ox*, were upregulated before and during anthesis in Satsuma ovary, coinciding with the onset of cell division and ovary growth, whereas they were downregulated in the Clemenules ovary. It is important to note that in the latter GA biosynthetic genes were upregulated when the abscission wave (15 DAA) is completed, i.e. this upregulation did not control the fruit set process but rather the fruitlet development that followed. The second wave of abscission, which only implies fruitlets not flowers, occurs about 4 weeks after anthesis (Agustí *et al*., 1982). Thus, the Clemenules mandarin lacks the constitutive activation of GA synthesis at anthesis that determines fruit set.

The phylogenetic analysis revealed two EST putative homolog to *CcGA20ox1* and *CcGA20ox2* genes in the *C. clementina* genome. Since *CcGA20ox2* is the one expressed in the pistil (Huerta *et al.,* 2009), we only studied the corresponding putative homolog in *C. clementina* (ciclev10020694m, named *GA20ox2* in this PhD-

Thesis). To our knowledge, this is the first study into *GA3ox* expression during citrus fruit development. Gibberellin 3-oxidase, in *Arabidopsis*, is comprised of a multigene family consisting of at least 4 members (Mitchum *et al.,* 2006). In general, the expression patterns of the *GA3ox* genes suggest that *AtGA3ox1* and *AtGA3ox2* are the major genes required for germination and vegetative growth, and that *AtGA3ox1*, *AtGA3ox3* and *AtGA3ox4* are essential for the development of reproductive organs (Mitchum *et al*., 2006). Using the protein of these four genes as a reference, we found 5 ESTs among the *GA3ox* family genes in the sequenced genome of *C. clementina*. All 5 ESTs belonged to the 2OG-Fe (II) oxygenase superfamily, to which the *GAox* enzymes belong. A phylogenetic tree was constructed to evaluate the evolutionary relationships among *GA3ox* amino acid sequences in *Arabidopsis thaliana*, *C. clementina* and other species (i.e. *Vitis vinifera*). For gene expression studies, and attending to the phylogenetic analysis, we selected two ESTs, Ciclev10027153m [*Citrus clementina*], named *GA3ox1* in this PhD-Thesis, which clustered with *VvGA3ox1* and was close to *AtGA3ox1*, *AtGAox2* and *AtGAox4*, and Ciclev10010629m [*Citrus clementina*], named *GA3ox2* in this Thesis, which clustered with *VvGA3ox2* and was close to the *AtGA3ox3* gene. Han *et al*. (2011) determined very small distance inside the *GA3ox* group between species, which implies that fewer sequence changes have occurred in *GA3ox* that might be under greater pressure to be conserved in comparison to the other GA-oxidases. In the experiments carried out here, *GA3ox* activity was produced for different genes depending on the species, *GA3ox2* and *GA3ox1* for Satsuma and Clemenules ovaries, respectively. These results do not disagree to that of other species. For instance, *AtGA3ox1* and *AtGA3ox3* are mainly expressed during the development of pollinated siliques, having overlapping roles, while *AtGA3ox2* and *AtGA3ox4* play minor roles (Hu *et al.,* 2008; Dorcey *et al.,* 2009). On the other hand, *VvGA3ox2* is expressed in the growing flower 1 DAA while *VvGA3ox1* is low at anthesis peaking 7 days later in the growing fruitlet (Giacomelli *et al.,* 2013). These results in Clemenules ovary regarding *GA3ox1* were similar to the latter. Finally, in wild type tomato, *SlGA3ox1* and *SlGA3ox2* were mainly detectable at preanthesis but also during induced parthenocarpic fruit development (Serrani *et al.,* 2008 and 2010). However, in the natural parthenocarpic *pat* mutant only *SlGA3ox2* was detectable (Ollimpieri *et al.,* 2007). The Satsuma ovary displayed similar results to that of *pat* tomato, but not the

Clemenules ovary, suggesting specific *GA3ox2* activity for parthenocarpic development.

The *GA2ox* gene expressions were alternatively upregulated and downregulated in both Clemenules and Satsuma within different time frames, but no conclusive data can be obtained in relation to parthenocarpy.

Overall, the expression data indicate that GA biosynthesis mediates parthenocarpic fruit set. Although a role for *GA3ox* and *GA2ox* genes is not completely ruled out, these results suggest that the transcriptional activation of *GA20ox2* represents a major regulatory switch for parthenocarpy.

*GA regulates cell division in the pericarp and the growing juice sacs*

The role of GA regulating cell proliferation was reported in the context of shoot elongation of the herbaceous plant *Silene armenia*, the data indicating a spatial correlation between the accumulation of $GA_1$ and the enhanced mitotic activity that occurs in the subapical meristem (Talón *et al*., 1991). In *Citrus*, the shorter and longer internodes produced by antisense and sense expression of *CcGA20ox1*, respectively, seem to be due to repression and induction of cell division rather than cell elongation (Fagoaga *et al.,* 2007). Regarding root and leaf growth, recent studies show that GA synthesis and signalling affect the size of the division zone resulting in proportional changes in organs, (i.e. root and leaf) and growth rates (Ubeda-Tomás *et al.,* 2009; Nelissen *et al*., 2012). To our knowledge, there is not any reference linking GA endogenous regulation of cell division during natural parthenocarpic fruit growth. Several findings from the experiments of the Thesis indicate that the specific GA synthesis in the pericarp is responsible for activating cell division, and thus parthenocarpy. This hypothesis is supported by 1) the hybridization analysis of the *GA20ox2* transcripts revealed weak expression of the gene in the Clemenules ovary at anthesis and restricted to the exocarp, while a strong hybridization signal was observed in the whole Satsuma ovary at the corresponding stage, thus correlating with the mitotic rate; 2) in Satsuma mandarin GA biosynthesis, i.e. *GA20ox-2* transcripts *in situ* localization, is first located in the entire pericarp at anthesis, but 30 days later it is specifically restricted to the endocarp and growing juice sacs where cell division is still

active, and not in the mesocarp where cells are not dividing but enlarging (Guardiola *et al*., 1993); 3) *CycA1,1* expression was upregulated when *GA20ox2* achieved its maximum expression rate at anthesis; 4) treatment with $GA_3$ and PBZ enhanced and impaired, respectively, *CycA1,1* expression and cell division in the ovary through modification of the expression of GAs metabolism genes and $GA_1$ concentration; and 5) *CycA1,1* expression correlated endogenous $GA_1$ content due to $GA_3$ treatment. The relationship between $GA_3$ exogenous application and *CycA1,1* was previously observed in rice (Fabian *et al.,* 2000). But in our experiments, the time-course of $GA_1$ endogenous concentration and *CycA1,1* gene expression strictly paralleled as a result of $GA_3$ application. In the Satsuma ovary this effect was temporarily, due to its natural ability to synthesize $GA_1$, and fruit growth did not disagree with untreated ovaries. But in the Clementine ovary this effect promotes fruit set and growth due to its low ability to synthesize $GA_1$.

According to the aforementioned, we conclude that GAs are responsible for cell division during fruit set in *Citrus* (Fig. 29). At the preanthesis stage, Satsuma and Clemenules ovary size and their number of cell layers in the pericarp are similar. But upregulation of biosynthetic *GAox* activity in the Satsuma pericarp before anthesis, compared to downregulation of these enzymes in the Clementine pericarp, leads to a higher $GA_1$ concentration, a higher cell division rate, a larger cell division zone, a higher fruit growth rate and, therefore, a higher parthenocarpic ability and fruit set in the former than in the latter. Afterwards, at the exponential fruitlet growth stage, GA biosynthesis is strictly restricted to the cell division zone in the pericarp, mainly in the endocarp where growing juice sacs start to emerge and grow (Fig. 19). At this stage, the expansion zone is located in the adjacent milimeters (i.e. the mesocarp in the citrus fruit) where IAA metabolism may be prevalent (Nelissen *et al.,* 2012).

Overall, the autonomous activation of GA biosynthesis in the pericarp tissues stimulates cell division at anthesis in *Citrus*. Maintaining a suitable GA concentration in the pericarp allows an active mitotic activity determining ovary growth and parthenocarpic fruit set.

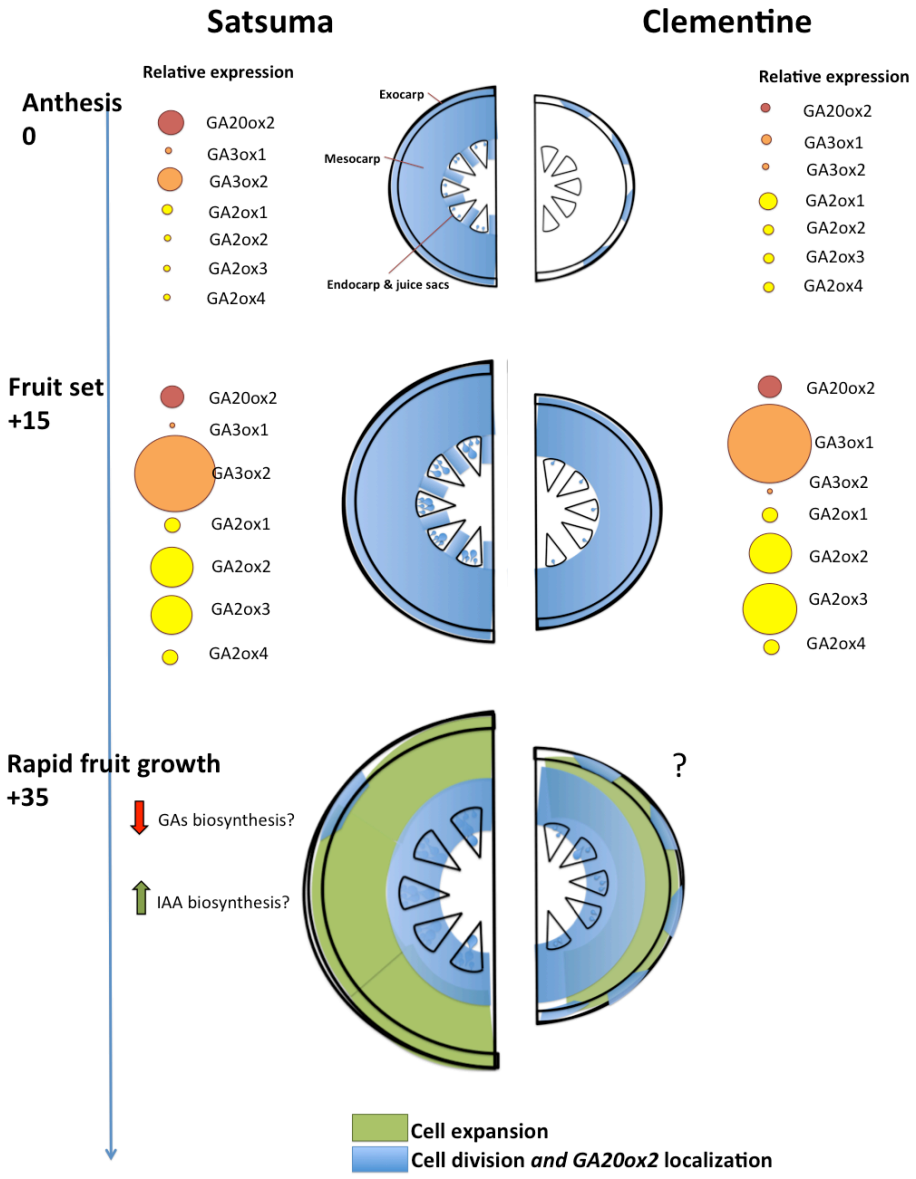



**Figure 29.** Visual summary of *GA20ox2*, *GA3ox1*, *GA3ox2* and *GA2ox-1,2,3,4* relative expression, *GA20ox2* localization '*in situ*', cell expansion and cell division in Satsuma mandarin (left) and Clementine mandarin (right) ovaries during the onset of fruiting (anthesis, 15 days after anthesis and 35 days after anthesis). Data are means ± ES of 3 qRT-PCR replicates.

But this autonomous activation of GA biosynthesis was found in the sterile Satsuma mandarin but not in the fertile and self-incompatible Clementine cv. Clemenules mandarin suggesting that autonomous parthenocarpy is a privative process of sterile genotypes. However, other fertile self-incompatible cultivars have the ability to set a high proportion of parthenocarpic fruits. In these cultivars, pollen grains are able to germinate in the stigma but pollen tubes are arrested in the style avoiding self-fertilization. Thus, the question is raised as to whether parthenocarpy is autonomous or stimulated in non-sterile citrus species. The hypothesis that self-incompatible Clementine mandarins present pollination-independent parthenocarpy was examined. To this end, the effects of self-pollination on $GA_1$ and $GA_4$ contents in the ovary were identified during fruit set of two Clementine mandarin cultivars, 'Clemenules' and 'Marisol', which differ in terms of parthenocarpic ability.

*Self-pollination do not initiates parthenocarpy in the Clementine mandarin*

In these experiments, self-pollination was not necessary to trigger parthenocarpic fruit development, whereas un-pollinated ovaries restarted growth as did self-pollinated ovaries; these grew up to 18- and 12-fold in 'Marisol' and 'Clemenules', respectively, during 45 days. In *Prunus* and *Citrus* seeded varieties, in which pollination and fertilization are absolutely necessary for fruit set, ovary growth of un-pollinated flowers was also observed in the first 2 weeks following anthesis. Thereafter, a rapid 100% ovary abscission was produced (Ben-Cheikh *et al*., 1997; Rodrigo and Herrero, 2002). Recently, Distefano *et al.* (2011) reported anatomical changes in the ovary occurring in the same way and at the same time in un-pollinated or cross-pollinated flowers of the self-incompatible 'Nova mandarin' (*C. clementina* Hort. ex Tan. × *C. reticulata* Blanco), which reflects an uncoupling of the fertilization and fruiting time processes. Thus, these findings indicate that ovary growth at the onset of the cell division stage is independent of pollination and, therefore, developmentally controlled (Rodrigo and Herrero, 2002; Distefano *et al.,* 2011).

Accordingly, these results also indicate that differences in the parthenocarpic ability of Clementine mandarin varieties are due to autonomous $GA_1$ levels in the ovary during anthesis. We found a significantly higher $GA_1$ content in the 'Marisol' ovary than

in the 'Clemenules' ovary, which is in accordance with the former's higher parthenocarpic ability. Talón *et al.* (1992) obtained similar results after comparing citrus species differing in their parthenocarpic ability, such as Satsuma cv. Okitsu mandarin (*C. unshiu* Marc.) and Clementine cv. Oroval mandarin (*C. clementina* Hort. ex Tan). Mesejo *et al.* (2008) suggested an inverse relationship between GA content in the ovary and citrus ovule longevity. The present results support this hypothesis, revealing a higher $GA_1$ concentration in the ovary of 'Marisol' mandarin that paralleled a higher ovule abortion. These results also agree with the pre-blossom applications of $GA_3$, which cause early ovule abortion in sweet cherry (Beppu *et al.,* 2001), grape (Kimura *et al.,* 1996) and citrus (Mesejo *et al.,* 2008). Further, our results are in agreement with the hypothesis that the autonomous increase in $GA_1$ ovary content during anthesis acts as a stimulus, thus participating in the process of the parthenocarpic transition from ovary to developing fruitlet (Talón *et al*., 1992).

In these experiments, self-pollination of self-incompatible flowers did not increase GA content in the ovary during fruit set. This result contrasts with those obtained by Ben-Cheikh *et al.* (1997) pollinating the self-compatible seeded 'Pineapple' sweet orange, which, as mentioned before, requires self-pollination to set. In seeded varieties, both pollination and seed-derived signals are required for fruit initiation and subsequent development (Wang *et al*., 2009). Nonetheless, in seedless varieties fruit set can be either pollination-dependent (Schijlen *et al*., 2007) or pollination-independent (Wang *et al*., 2009). In the latter, an increased level of hormones (i.e. GA) in the ovary can substitute pollination and trigger fruit development (Gorguet *et al.,* 2005), which supports our results.

The involvement of a GA-mediated control of photosynthesis by the fruit is possible because $GA_3$ applications have been shown to increase photosynthetic rate by promoting Rubisco activity (Yuan and Xu, 2001), and the increased GA content in transgenic Carrizo citrange (*C. sinensis* × *P. trifoliata*) induced overall upregulation of the genes involved in photosynthetic and carbon utilization (Huerta *et al*., 2008). In our experiments, however, photosynthetic rate (PSII) of the leaves adjacent to the fruit was not significantly different for 'Marisol' and 'Clemenules' during the first wave of fruitlet abscission (20 DAP), but it was during the second wave (33 DAP) (data not

shown), coinciding with the leaf sink-to-source transition (Rivas *et al.,* 2007). On the other hand, the fruit's own photoassimilate production has been suggested to play an important role in tomato fruit establishment (Kolotilin *et al*., 2007; Wang *et al*., 2009), because transcriptional upregulation of photosynthesis has been observed during pollination-dependent and independent tomato fruit set (Wang *et al*., 2009). The relationship between GA content in the ovary and fruit photosynthesis activation during the flower-to-fruit transition deserves more attention.

Carbohydrate reserves within the ovary diminish rapidly following anthesis, given the high metabolic activity and high rate of cell division, paralleling ovary growth (Mehouachi *et al*., 1995; Rodrigo *et al*., 2000). Since this process takes place in both pollinated and un-pollinated ovaries, even in pollination-dependent species (Rodrigo and Herrero, 2002), it seems to be inherent to the flower and not dependent on pollination (Rodrigo *et al*., 2000; Wang *et al.,* 2009). Furthermore, differences were not significant for starch and hexoses ovary concentrations for un-pollinated and self-pollinated Clementine flowers of both cultivars. On the other hand, the larger hexoses consumption of 'Marisol' fruitlets in comparison with 'Clemenules' fruitlets paralleled the higher fruitlet growth rate and higher $GA_1$ levels in the ovary of the former. In 'Marisol' mandarin, 5 days after pollination hexoses concentration increased by 25% and decreased sharply (70%) thereafter. In contrast, 'Clemenules' mandarin showed lower hexoses mobilization.

A lack of carbohydrates can reduce fruitlet growth rate and trigger ABA and ethylene synthesis causing fruitlet abscission (Mehouachi *et al*., 1995; Gómez- Cadenas *et al*., 2000; Iglesias *et al*., 2006; Mesejo *et al.,* 2012). Moreover, under normal growth conditions, ABA and ethylene are involved in inducing the abscission process, and directly related to the intensity of abscission (Zacarías *et al.,* 1995, Gómez-Cadenas *et al.*, 2000). The interaction between GA, ABA and starch metabolism has been studied in other experimental systems (Rogers and Rogers, 1992). GA induces the transcription of several genes encoding hydrolytic enzymes, including α-amylase, whereas ABA suppresses α-amylase expression (Weiss and Ori, 2007). In the experiments of this Thesis, this relationship was also observed at anthesis (high GA – low ABA – high starch hydrolysis, and vice-versa). Moreover, high ABA concentration was associated with reduced Clementine fruit growth and high abscission, whereas a single application of

$GA_3$ completely suppressed the ABA increase and also promoted the rate of growth (Zacarías *et al*., 1995), which is in accordance with the results presented here. The results conclude that starch hydrolysis, as well as GA – ABA ratio, are constitutively regulated during fruit set rather than controlled by self-pollination in the Clementine mandarin.

Considering the results presented herein, ovary IAA concentration does not seem to be a limiting or controlling factor in early fruit development in Clementine mandarin, as previously reported for sweet orange (Talón *et al.,* 1990). On the other hand, polar auxin transport from the fruit to the pedicel prevents early fruitlet abscission, and a decline in polar auxin transport triggers abscission (Else *et al*., 2004; Blanusa *et al.,* 2005). IAA is an essential stimulation element in vascular tissue differentiation (Aloni, 2010), and a direct effect of the synthetic auxins promoting the development of citrus peduncle vascular tissue was demonstrated by applying the auxin locally to the peduncle (Mesejo *et al*., 2003). IAA also reduces the sensitivity of cells to ethylene in the abscission layers (Paterson, 2001). When comparing fruits with different abscission rates, Blanusa *et al.* (2005) found significant differences in PAT, but not in ovary IAA concentration. These authors suggested that the declining PAT capacity did not appear to be due to different concentrations of fruit IAA potentially available for PAT. Further studies are needed to explain the role of auxin in citrus fruitlet abscission.

In conclusion, (1) self-pollination did not alter ovule abortion rate, fruit set, fruit fresh weight or ovary GA content of either cultivar; (2) $GA_1$ and ABA ovary contents at anthesis were higher and lower, respectively, in ‘Marisol’ than in ‘Clemenules’ mandarin, and this paralleled the higher fruit set of the former; (3) ‘Marisol’ ovaries had greater starch and hexoses mobilization the days following anthesis, which also paralleled their higher $GA_1$ content. Consequently, we propose that the Clementine mandarin presents pollination-independent facultative parthenocarpy, rather than stimulative parthenocarpy, with its ability to set depending on endogenous $GA_1$ levels in the ovary that might be autonomously triggered, depending on the genotype.

# CONCLUSIONS

Attending to the tested hypothesis and the 3 objectives established in this Ph-D Thesis, the main findings are:

1. In the parthenocarpic *Citrus* cultivars GA biosynthesis is first located in the whole pericarp and the ovules during the flower to fruit transition; later, during the fruit cell division developmental stage, GA biosynthesis is located in the endocarp and growing juice sacs.

2. Difference in $GA_1$ concentration in the ovary between high and low parthenocarpic varieties is mainly determined by the autonomous upregulation of GA20ox2 enzyme rather than $GA_1$ irreversible deactivation by 2-hydroxylation (*GA2ox* activity).

3. The autonomous activation of GA biosynthesis directly stimulates *CYCA1,1* gene expression and cell division in the ovary walls (exocarp and mainly mesocarp) and in the endocarp (growing juice sacs), increasing ovary growth rates and leading to higher parthenocarpic fruit set.

4. The self-incompatible Clementine mandarin presents pollination-independent parthenocarpy, rather than stimulative parthenocarpy, with its ability to set being dependent on endogenous $GA_1$ levels in the ovary at anthesis, which is triggered autonomously depending on the genotype.

# REFERENCES

**Agustí, J., Zapater, M., Iglesias, D. J., Cercós, M., Tadeo, F. R., & Talón, M. (2007).** Differential expression of putative 9-*cis*-epoxycarotenoid dioxygenases and abscisic acid accumulation in water stressed vegetative and reproductive tissues of citrus. *Plant Science*, *172*(1), 85-94.

**Agustí, J., Merelo, P., Cercós, M., Tadeo, F. R., & Talón, M. (2008).** Ethylene-induced differential gene expression during abscission of citrus leaves. *Journal of Experimental Botany*, *59*(10), 2717-2733.

**Agustí, M., Garcia-Mari, F., & Guardiola, J. L. (1982).** Gibberellic acid and fruit set in sweet orange. *Scientia horticulturae*, 17(3), 257-264.

**Agustí, M., Zaragoza, S., Bleiholder, H., Buhr, L., Hack, H., Klose, R., & Stauβ, R. (1997).** Adaptation of the BBCH scale for the description of Citrus fruits' phenological stages. *Fruits*, *52*(5), 287-295.

**Agustí, M., Martinez-Fuentes, A., & Mesejo, C. (2002).** Citrus fruit quality. Physiological basis and techniques of improvement. *Agrociencia*, *6*(2), 1-16.

**Agustí, M. (2003).** *Citricultura*. Madrid: Ediciones Mundi-Prensa.

**Agustí, M., Mesejo, C., Reig, C., & Martínez-Fuentes, A. (2014).** Citrus production. In *Horticulture: Plants for People and Places, Volume 1* (pp. 159-195). Springer Netherlands.

**Albone, K. S., Gaskin, P., MacMillan, J., Phinney, B. O., & Willis, C. L. (1990).** Biosynthetic origin of gibberellin A3 and gibberellin A7 in cell-free preparations from seeds of Marahmacrocarpus and Malus domestica. *Plant Physiology*. 94, 132-142.

**Aloni, R. (2010).** The induction of vascular tissues by auxin. In: Davies PJ (ed) *Plant hormones*. Biosynthesis, signal transduction, action. Springer, Amsterdam, pp 485–506.

**Alquézar, B., Zacarías, L., & Rodrigo, M. J. (2009).** Molecular and functional characterization of a novel chromoplast-specific lycopene β-cyclase from Citrus and its relation to lycopene accumulation. *Journal of Experimental Botany*, 60(6), 1783-1797.

**Asahina, M., Iwai, H., Kikuchi, A., Yamaguchi, S., Kamiya, Y., Kamada, H., & Satoh, S. (2002).** Gibberellin produced in the cotyledon is required for cell division during tissue reunion in the cortex of cut cucumber and tomato hypocotyls. *Plant Physiology*, *129*(1), 201-210.

**Baluska, F., Parker, J. S., & Barlow, P. W. (1993).** A role for gibberellic acid in orienting microtubules and regulating cell growth polarity in the maize root cortex. *Planta*, 191(2), 149-157.

**Ben-Cheikh W., Pérez-Botella J., Tadeo F. R., Talón M., & Primo-Millo E. (1997).** Pollination increases gibberellin levels in developing ovaries of seeded varieties of citrus. *Plant Physiology* 114: 557-564.

**Beppu, K., Suehara, T., & Kataoka, I. (2001).** Embryo sac development and fruit set of 'Satonishiki'sweet cherry [Prunus avium] as affected by temperature, $GA_3$ and paclobutrazol. *Journal of the Japanese Society for Horticultural Science (Japan)*.

**Bieto, J. A., & Cubillo, M. T. (2008).** *Fundamentos de fisiología vegetal*. McGraw-Hill Interamericana de España.

**Blanusa, T., Else, M. A., Atkinson, C. J., & Davies, W. J. (2005).** The regulation of sweet cherry fruit abscission by polar auxin transport. *Plant growth regulation*, *45*(3), 189-198.

**Bonanda, B. R. (2011).** Anomalies in structure, growth characteristics, and nutritional composition as induced by 2,4-dichlorophenoxy acetic acid drift phytotoxicity in grapevine leaves and clusters. *Journal of the American Society for Horticultural Science.* 136: 165–176.

**Boss, P. K., & Thomas, M. R. (2002).** Association of dwarfism and floral induction with a grape 'green revolution'mutation. *Nature*, *416*(6883), 847-850.

**Brown, H. D., & Krezdorn, A. H. (1970).** Hand pollination tests and field evaluation of pollinators for citrus (Master's thesis, University of Florida).

**Calabrese, F. (2004).** La Favolosa Storia degli Agrumi. (The fascinating history of citrus fruit).L'EPOS Società Editrici s.a.s., Palermo.

**Carbonell-Bejerano, P., Urbez, C., Granell, A., Carbonell, J., & Perez-Amador, M. A. (2011).** Ethylene is involved in pistil fate by modulating the onset of ovule senescence and the GA-mediated fruit set in Arabidopsis. *BMC plant biology*,*11*(1), 84.

**Chai, L., Ge, X., Biswas, M. K., Xu, Q., & Deng, X. (2011).** Self-sterility in the mutant 'Zigui shatian'pummelo (Citrus grandis Osbeck) is due to abnormal post-zygotic embryodevelopment and not self-incompatibility. *Plant Cell, Tissue and Organ Culture* (PCTOC), 104(1), 1-11.

**De Jong, M., Mariani, C., & Vriezen, W. H. (2009).** The role of auxin and gibberellin in tomato fruit set. *Journal of experimental botany*, erp094.

**Del Pozo, J. C., Lopez-Matas, M., Ramirez-Parra, E., & Gutierrez, C. (2005).** Hormonal control of the plant cell cycle. *Physiologia Plantarum*, *123*(2), 173-183.

**Distefano, G., Gentile, A., & Herrero, M. (2011).** Pollen–pistil interactions and early fruiting in parthenocarpic citrus. *Annals of botany*, *108*(3), 499-509.

**Dorcey, E., Urbez, C., Blázquez, M. A., Carbonell, J., & Perez-Amador, M. A. (2009).** Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in Arabidopsis. *The Plant Journal*, *58*(2), 318-332.

**Ebadi, A., Rezaei, M., & Fatahi, R. (2010).** Mechanism of seedlessness in Iranian seedless barberry (< i> Berberis vulgaris</i> L. var. asperma). *Scientia horticulturae*, *125*(3), 486-493.

**El-Otmani, M., Lovatt, C. J., Coggins Jr, C. W., & Agustí, M. (1995).** Plant growth regulators in citriculture: factors regulating endogenous levels in citrus tissues. *Critical reviews in plant sciences*, *14*(5), 367-412.

**Else, M. A., Stankiewicz-Davies, A. P., Crisp, C. M., & Atkinson, C. J. (2004).** The role of polar auxin transport through pedicels of Prunus avium L. in relation to fruit development and retention. *Journal of experimental botany*, *55*(405), 2099-2109.

**Estornell, L. H., Agustí, J., Merelo, P., Talón, M., & Tadeo, F. R. (2013).** Elucidating mechanisms underlying organ abscission. *Plant Science*, *199*, 48-60.

**Eti, S. and Stosser, R. (1992)**. Pollen tube growth and development of ovules in relation to fruit set in mandarins, cv. 'Clementine' (*Citrus reticulata* Blanco). *Acta Horticulturae*. 321: 621-625.

**Fabian, T., Lorbiecke, R., Umeda, M., & Sauter, M. (2000).** The cell cycle genes cycA1; 1 and cdc2Os-3 are coordinately regulated by gibberellin in plant. *Planta*, *211*(3), 376-383.

**Fagoaga, C., Tadeo, F. R., Iglesias, D. J., Huerta, L., Lliso, I., Vidal, A. M., Talón, M., Navarro, L., García-Martínez, J.L., & Peña, L. (2007).** Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture. *Journal of experimental botany*, 58(6), 1407-1420.

**Food and Agriculture Organization (FAO),** http://faostat3.fao.org/browse/Q/QC/E

**Garcia-Papi, M. A., & Garcia-Martinez, J. L. (1984).** Endogenous plant growth substances content in young fruits of seeded and seedless Clementine mandarin as related to fruit set and development. *Scientia horticulturae*, *22*(3), 265-274.

**Giacomelli, L., Rota-Stabelli, O., Masuero, D., Acheampong, A. K., Moretto, M., Caputi, L., Vrhovsek, U., & Moser, C. (2013).** Gibberellin metabolism in Vitis vinifera L. during bloom and fruit-set: functional characterization and evolution of grapevine gibberellin oxidases. *Journal of experimental botany*, *64*(14), 4403-4419.

**Gillaspy, G., Ben-David, H., & Gruissem, W. (1993).** Fruits: a developmental perspective. *The Plant Cell*, 5(10), 1439.

**Glasziou, K. T., & Gayler, K. R. (1969).** Sugar transport: occurrence of trehalase activity in sugar cane. *Planta*, *85*(3), 299-302.

**Goldschmidt, E. E., & Monselise, S. P. (1977).** Physiological a ssumptions toward the development of a citrus fruiting model. *Methods*, *50*, 1.

**Gómez-Cadenas, A., Mehouachi, J., Tadeo, F. R., Primo-Millo, E., & Talon, M. (2000).** Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. *Planta*, *210*(4), 636-643.

**Gorguet, B., Heusden, A. V., & Lindhout, P. (2005).** Parthenocarpic fruit development in tomato. *Plant Biology*, *7*(2), 131-139.

**Guardiola, J. L., Barres, M. T., Albert, C., & García-Luis, A. (1993).** Effects of exogenous growth regulators on fruit development in Citrus unshiu. *Annals of botany*, 71(2), 169-176.

**Guardiola, J. L., García-Marí, F., & Agustí, M. (1984).** Competition and fruit set in the Washington navel orange. *Physiologia Plantarum*, *62*(3), 297-302.

**Han, F., & Zhu, B. (2011).** Evolutionary analysis of three gibberellin oxidase genesin rice, Arabidopsis, and soybean. *Gene*, *473*(1), 23-35.

**Hanania, U., Velcheva, M., Or, E., Flaishman, M., Sahar, N., & Perl, A. (2007).** Silencing of chaperonin 21, that was differentially expressed in inflorescence of seedless and seeded grapes, promoted seed abortion in tobacco and tomato fruits. *Transgenic research*, *16*(4), 515-525.

**Hedden, P., & Phillips, A. L. (2000).** Gibberellin metabolism: new insights revealed by the genes. *Trends in plant science*, *5*(12), 523-530.

**Hernández Miñana, F. M., & Primo-Millo, E. (1990).** Studies on endogenous cytokinins in Citrus. *Journal of Horticultural Science*, *65*(5), 595-601.

**Hu, J., Barlet, X., Deslandes, L., Hirsch, J., Feng, D. X., Somssich, I., & Marco, Y. (2008).** Transcriptional responses of Arabidopsis thaliana during wilt disease caused by the soil-borne phytopathogenic bacterium, Ralstonia solanacearum. *PLoS One*, *3*(7), e2589.

**Huerta, L., Forment, J., Gadea, J., Fagoaga, C., Pena, L., Pérez-Amador, M. A., & García-Martínez, J. L. (2008).** Gene expression analysis in citrus reveals the role of gibberellins on photosynthesis and stress. *Plant cell & environment*, *31*(11), 1620-1633.

**Huerta, L., Garcia-Lor, A., & Garcia-Martinez, J. L. (2009).** Characterization of gibberellin 20-oxidases in the citrus hybrid Carrizo citrange. *Tree physiology*, *29*(4), 569-577.

**Iglesias, D. J., Tadeo, F. R., Primo-Millo, E., & Talon, M. (2006).** Carbohydrate and ethylene levels related to fruitlet drop through abscission zone A in citrus. *Trees*, *20*(3), 348-355.

**Inzé, D., & De Veylder, L. (2006).** Cell cycle regulation in plant development. *Annual Review of Genetics.*, *40*, 77-105.

**Iwamasa, M. (1966).** Studies on the sterility on genus citrus with special reference to seedlessness. *Bulletin of the Horticultural Research Station in Japan,* B 6: 1–77.

**Jackson, D.P. (1992).** *In situ hybridization* in plants. In Molecular Plant Pathology: A practical approach (DJ Bowles, SJ. Gurr & M.McPhereson, Eds) Oxford University Press, Oxford, pp. 163-174.

**Kimura, P. H., Okamoto, G., & Hirano, K. (1996).** Effects of gibberellic acid and streptomycin on pollen germination and ovule and seed development in Muscat Bailey A. *American journal of enology and viticulture*, *47*(2), 152-156.

**King, R. W., & Ben-Tal, Y. (2001).** A florigenic effect of sucrose in Fuchsia hybrida is blocked by gibberellin-induced assimilate competition. *Plant Physiology*, *125*(1), 488-496.

**Knight, T. G., Klieber, A., & Sedgley, M. (2001).** The relationship between oil gland and fruit development in Washington navel orange (Citrus sinensis L. Osbeck). *Annals of Botany*, *88*(6), 1039-1047.

**Kojima, K., Yamamoto, M., Goto, A., & Matsumoto, R. (1996).** Changes in ABA, IAA and GAs contents in reproductive organs of satsuma mandarin. *Journal of the Japanese Society for the Horticultural Science.* 65: 237-243.

**Kolotilin, I., Koltai, H., Tadmor, Y., Bar-Or, C., Reuveni, M., Meir, A., ... & Levin, I. (2007).** Transcriptional profiling of high pigment-2dg tomato mutant links early fruit plastid biogenesis with its overproduction of phytonutrients. *Plant physiology*, *145*(2), 389-401.

**Krezdorn, A. H. (1970).** Pollination requirements of citrus. In the Indispensable Pollinators, Ark. Agr. Ext. Serv. Misc. Pub. 127, pp. 211-218.

**Lange, T., Hedden, P., & Graebe, J. E. (1994).** Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. *Proceedings of the National Academy of Sciences*, *91*(18), 8552-8556.

**Linskens, H. F., & Esser, K. (1957).** Uber eine spezifische Anfarbung der Pollenschfauche und die zahl kallosapropfen nach Selbstung und Fremdung.*Naturwissenschaften 44*, *16*.

**Livak, K. J., & Schmittgen T. D. (2001).** Analysis of relative gene expression data using real-time quantitative PCR and the 2(- Delta Delta C(T)) Method. *Methods* 25, 402–8.

**Seo, M., Jikumaru, Y., & Kamiya, Y. (2011)**. Profiling of Hormones and Related Metabolites in Seed Dormancy and Germination Studies. *Methods in Molecular Biology* 773: 99-111.

**MacMillan, J., & Takahashi, N. (1968).** Proposed procedure for the allocation of trivial names to the gibberellins.

**Mafra, V., Kubo, K. S., Alves-Ferreira, M., Ribeiro-Alves, M., Stuart, R. M., Boava, L. P., Rodrigues, C. M., & Machado, M. A. (2012).** Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PloS one*, 7(2), e31263.

**Malladi, A., & Johnson, L. K. (2011).** Expression profiling of cell cycle genes reveals key facilitators of cell production during carpel development, fruit set, and fruit growth in apple (Malus× domestica Borkh). *Journal of experimental botany*, *62*(1), 205-219.

**Martínez-Fuentes, A., Mesejo, C., Reig, C., & Agustí, M. (2010).** Timing of the inhibitory effect of fruit on return bloom of 'Valencia'sweet orange (Citrus sinensis (L.) Osbeck). *Journal of the Science of Food and Agriculture*, *90*(11), 1936-1943.

**Mauk, C. S., Bausher, M. G., & Yelenosky, G. (1986).** Influence of growth regulator treatments on dry matter production, fruit abscission, and 14C-assimilate partitioning in citrus. *Journal of Plant Growth Regulation*, *5*(2), 111-120.

**McClure, B. A., Gray, J. E., Anderson, M. A., & Clarke, A. E. (1990).** Self-incompatibility in Nicotiana alata involves degradation of pollen rRNA. *Nature*,*347*(6295), 757-760.

**Mehouachi, J., Serna, D., Zaragoza, S., Agustí, M., Talón, M., & Primo-Millo, E. (1995).** Defoliation increases fruit abscission and reduces carbohydrate levels in developing fruits and woody tissues of Citrus unshiu. *Plant Science,* 107: 189–197.

**Mesejo, C., Martínez-Fuentes, A., Juan, M., Almela, V., & Agustí, M. (2003).** Vascular tissues development of citrus fruit peduncle is promoted by synthetic auxins. *Plant growth regulation*, *39*(2), 131-135.

**Mesejo, C., Martínez-Fuentes, A., Reig, C., & Agustí, M. (2007).** The effective pollination period in 'Clemenules' mandarin, 'Owari' Satsuma mandarin and 'Valencia' sweet orange. *Plant science*, *173*(2), 223-230.

**Mesejo, C., Martínez-Fuentes, A., Reig, C., & Agustí, M. (2008).** Gibberellic acid impairs fertilization in Clementine mandarin under cross-pollination conditions. *Plant science*, *175*(3), 267-271.

**Mesejo, C., Rosito, S., Reig, C., Martínez-Fuentes, A., & Agustí, M. (2012).** Synthetic auxin 3, 5, 6-TPA provokes citrus clementina (Hort. ex Tan) fruitlet abscission by reducing photosynthate availability. *Journal of Plant Growth Regulation*, *31*(2), 186-194.

**Mesejo, C., Muñoz-Fambuena, N., Reig, C., Martínez-Fuentes, A., & Agustí, M. (2014).** Cell division interference in newly fertilized ovules induces stenospermocarpy in cross-pollinated citrus fruit. *Plant Science*.

**Mitchum, M. G., Yamaguchi, S., Hanada, A., Kuwahara, A., Yoshioka, Y., Kato, T., ... & Sun, T. P. (2006).** Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. *The Plant Journal*, 45(5), 804-818.

**Monselise, S. P. (1977).** Citrus fruit development: endogenous systems and external regulation. Proceedings of the International Society of Citriculture. (Vol. 2, pp. 664-668).

**Moubayidin, L., Perilli, S., Dello Ioio, R., Di Mambro, R., Costantino, P., & Sabatini, S. (2010).** The Rate of Cell Differentiation Controls the *Arabidopsis* Root Meristem Growth Phase. *Current Biology*, *20*(12), 1138-1143.

**Nelissen, H., Rymen, B., Jikumaru, Y., Demuynck, K., Van Lijsebettens, M., Kamiya, Y., & Beemster, G. T. (2012).** A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. *Current Biology*, 22(13), 1183-1187.

**Olimpieri, I., Siligato, F., Caccia, R., Soressi, G. P., Mazzucato, A., Mariotti, L., & Ceccarelli, N. (2007).** Tomato fruit set driven by pollination or by the parthenocarpic fruit allele are mediated by transcriptionally regulated gibberellin biosynthesis. *Planta*, *226*(4), 877-888.

**Paterson, SE. (2001).** Cutting loose. Abscission and dehiscence in *Arabidopsis*. *Plant Physiology,* 126:494–500

**Phillips, A.L., Ward, D.A., Uknes, S., Appleford, N.E.J., Lange, T., Huttly, A.K., Gaskin, P., Graebe, J.E., & Hedden, P. (1995).** Isolation and expression of three gibberellin 20-oxidase cDNA clones from Arabidopsis. *Plant Physiology*, 108, 1049 1097.

**Phinney, B.C. (1984).** Gibberellin $A_1$, dwarfism and the control of shoot elongation in higher plants. In: *The biosynthesis and metabolism of plant hormones*, pp. 17–41, Crozier, A., Hillman, J.R., eds. Cambridge University Press, Cambridge, UK.

**Pons, J., Pastor, J., Polls, M., & Reverter, A. J. (1996)**. Polinización cruzada en cítricos III. Polinización entomófila. Efecto de repelentes. *Levante Agrícola*. 337: 291-295.

**Rieu, I., Eriksson, S., Powers, S. J., Gong, F., Griffiths, J., Woolley, L., Benlloch, R., Nilsson, O., Stephen, G. T., Hedden, P., & Phillips, A. L. (2008a).** Genetic analysis reveals that C19-GA 2-oxidation is a major gibberellin inactivation pathway in Arabidopsis. *The Plant Cell Online*, *20*(9), 2420-2436.

**Rieu, I., Ruiz-Rivero, O., Fernandez-Garcia, N. et al. (2008b).** The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. *Plant Journal*. 53, 488–504.

**Rivas, F., Erner, Y., Alós, E., Juan, M., Almela, V., & Agustí, M. (2006).** Girdling increases carbohydrate availability and fruit-set in citrus cultivars irrespective of parthenocarpic ability. *Journal of Horticultural Science and Biotechnology*,*81*(2), 289-295.

**Rivas, F., Gravina, A., & Agustí, M. (2007).** Girdling effects on fruit set and quantum yield efficiency of PSII in two Citrus cultivars. *Tree physiology*, *27*(4), 527-535.

**Rivero, J. M., Veyrat, P., & Gómez de Barreda, D. (1968).** Improving fruit set in Clementine mandarin with chemical treatments in Spain. *Proceedings of the First Interntational Citrus Symposium*., 3: 1121-1124**.**

**Rodrigo, M. J., García-Martínez, J. L., Santes, C. M., Gaskin, P., & Hedden, P. (1997).** The role of gibberellins A1 and A3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A4 and A7 in young seeds. *Planta*, 201(4), 446-455.

**Rodrigo, M. J., Hormaza, J. I., & Herrero, M. (2000).** Ovary starch reserves and flower development in apricot (*Prunus armeniaca*). *Physiologia plantarum*,108(1), 35-41.

**Rodrigo, M. J., & Herrero, M. (2002).** Effects of pre-blossom temperatures on flower development and fruit set in apricot. *Scientia Horticulturae*, *92*(2), 125-135.

**Rodrigo, M. J., Marcos, J. F., & Zacarías, L. (2004).** Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (Citrus sinensis L.) during fruit development and maturation. *Journal of Agricultural and Food Chemistry*; 52:6724-6731.

**Rogers, J. C., & Rogers, S. W. (1992).** Definition and functional implications of gibberellin and abscisic acid cis-acting hormone response complexes. *The Plant Cell Online*, *4*(11), 1443-1451.

**Sagee, O., & Erner, Y. (1991).** Gibberellins and abscisic acid contents during flowering and fruit set of 'Shamouti'orange. *Scientia horticulturae*, *48*(1), 29-39.

**Saito A, Fukasawa AT, Igarashi M, Sato T, Suzuki M. (2007).** Self-compatibility of 3 apple *Malus pumila* cultivars and identification of S-allele genotypes in their self-pollinated progenies. *Horticultural Research,* 6: 27–32.

**Schijlen, E. G., de Vos, C. R., Martens, S., Jonker, H. H., Rosin, F. M., Molthoff, J. W., & Bovy, A. G. (2007).** RNA interference silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. *Plant Physiology*, *144*(3), 1520-1530.

**Schneider, H. (1968).** Anatomy of citrus. *Reuther, W. The citrus industry*, *2*.

**Schomburg, F. M., Bizzell, C. M., Lee, D. J., Zeevaart, J. A. D., & Amasino, R. M. (2003).** Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. *Plant Journal*. 14, 1-14.

**Scofield, S., Jones, A., & Murray, J. A. (2014).** The plant cell cycle in context. *Journal of Experimental Botany*, *65*(10), 2557-2562.

**Serfontein CM, Catling HD. 1968.** Determining the canopy area of citrus trees. *South African Citrus Journal* 413: 14-15.

**Serrani, J. C., Carrera, E., Ruiz-Rivero, O., Gallego-Giraldo, L., Peres, L. E., & Garcia-Martinez, J. L. (2010).** Inhibition of auxin transport from the ovary or from the apical shoot induces parthenocarpic fruit-set in tomato mediated by gibberellins. *Plant physiology*, pp 110.

**Serrani, J. C., Ruiz-Rivero, O., Fos, M., & García-Martínez, J. L. (2008).** Auxin-induced fruit-set in tomato is mediated in part by gibberellins. *The Plant Journal*,*56*(6), 922-934.

**Serrani, J. C., Sanjuán, R., Ruiz-Rivero, O., Fos, M., & García-Martínez, J. L. (2007).** Gibberellin regulation of fruit set and growth in tomato. *Plant physiology*, *145*(1), 246-257.

**Soost, R. K., & Burnett, R. H. (1961).** Effects of gibberellin on yield and fruit characteristics of Clementine mandarin. Proceedings of the American Society for Horticultural Science. Vol. 77, pp. 194-201.

**Spiegel-Roy, P., & Goldschmidt, E. E. (1996).** *The biology of citrus*. Cambridge University Press.

**Sponsel, V. M., & Hedden, P. (2010).** Gibberellin biosynthesis and inactivation. *Plant Hormones*, 63-94.

**Spray, C. R., Kobayashi, M., Suzuki, Y., Phinney, B. O., Gaskin, P., & Macmillan, J. (1996).** The dwarf-1 (d1) mutant of Zea mays blocks 3 steps in the gibberellin-biosynthetic pathway. *Proceedings of the National Academy of Science of the United States of America.* 93, 10515-10518.

**Striem, M. J., Spiegel-Roy, P., Baron, I., & Sahar, N. (1992).** The degrees of development of the seed-coat and the endosperm as separate subtraits of stenospermocarpic seedlessness in grapesl). *Vitis*, *31*, 149-155.

**Swingle, W. T. (1967).** The botany of Citrus and its wild relatives. *The citrus industry*, 190-430.

**Tadeo, F. R., Primo-Millo, E. (1998).** An ultrastructural study on development and degeneration of unfertilized Citrus ovules, in: Proceedings of the Sixth International Citrus Congress, 1998, pp. 431–441.

**Takahashi, N., Yamaguchi, I., Kono, T., Igoshi, M., Hirose, K., & Suzuki, K. (1975).** Characterization of plant growth substances in Citrus unshiu and their change in fruit development. *Plant and Cell Physiology*, *16*(6), 1101-1111.

**Talon, M., Tadeo, F. R., & Zeevaart, J. A. (1991).** Cellular changes induced by exogenous and endogenous gibberellins in shoot tips of the long-day plant Silene armeria. *Planta*, *185*(4), 487-493.

**Talon, M., Zacarias, L., & Primo-Millo, E. (1992).** Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. *Plant physiology*, 99(4), 1575-1581.

**Talón, M., 2000**. Giberelinas. In : Azcón.Bieto, J., and Talón, M., Eds. Fundamentos de Fisiología Vegetal. McGraw-Hill, Universitat de Barcelona, Barcelona, Spain, pp 325-341.

**Tanaka, T. (1977).** Fundamental discussion of Citrus classification. *Stud Citrol*,*14*, 1-6.

**Tantikanjana, T., Nasrallah, M. E., & Nasrallah, J. B. (2010).** Complex networks of self-incompatibility signaling in the Brassicaceae. *Current opinion in plant biology*, *13*(5), 520-526.

**Thomas, S. G., Phillips, A. L., & Hedden, P. (1999).** Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proceedings of the National Academy of Science of the United States of America.* 96, 4698-4703.

**Ubeda-Tomás, S., Federici, F., Casimiro, I., Beemster, G. T., Bhalerao, R., Swarup, R., Doerner, P., Haseloff, J., & Bennett, M. J. (2009).** Gibberellin Signaling in the Endodermis Controls *Arabidopsis* Root Meristem Size. *Current Biology*, *19*(14), 1194-1199.

**Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., & Hanada, A. (2007).** Methylation of gibberellins by Arabidopsis GAMT1 and GAMT2. *Plant Cell*, in press.

**Vardi, A., Shani, A. F., & Weinbaum, S. A. (1988).** Assessment of parthenocarpic tendency in citrus using irradiated marker pollen. *Proc 6th Intl Citrus Congress* 1: 225–230.

**Vardi, A., Levin, I., & Carmi, N. (2008).** Induction of seedlessness in citrus: from classical techniques to emerging biotechnological approaches. *Journal of American Society for the Horticulture Science* 133:117–126.

**Varoquax, F., Blanvillain, R., Delseny, M., & Gallois, P. (2000).** Less is better: new approaches for seedless fruit production. *Trends Biotechnology* 18: 233–242.

**Wang, H., Schauer, N., Usadel, B., Frasse, P., Zouine, M., Hernould, M., & Bouzayen, M. (2009).** Regulatory features underlying pollination-dependent and-independent tomato fruit set revealed by transcript and primary metabolite profiling. *The Plant Cell Online,* 21(5), 1428-1452.

**Ward, J. L., Jackson, G. J., Beale, M. H., Gaskin, P., Hedden, P., Mander, L. N., Phillips, A. L., Seto, H., Talon, M., Willis, C. L., Wilson, T. M., & Zeevaart, J. A. D. (1997).** Stereochemistry of the oxidation of gibberellin 20-alcohols, $GA_{15}$ and $GA_{44}$, to 20-aldehydes by gibberellin 20-oxidases. *Chemical. Communications*. 13-14.

**Weiss, D., & Ori, N. (2007).** Mechanisms of cross talk between gibberellin and other hormones. *Plant Physiology. 144*(3), 1240-1246.

**Wu, G. A., Prochnik, S., Jenkins, J., Salse, J., Hellsten, U., Murat, F., Perrier, X., Ruiz, M., Scalabrin, S., Terol, J., Takita, M. A., Labadie, K., Poulain, J., Couloux, A., Jabbari, K., Cattonaro, F., Del Fabbro, C., Pinosio, S., Zuccolo, A., Chapman, J., Grimwood, J., Tadeo, F. R., Estornell, L. H., Muñoz-Sanz, J. V., Ibanez, V., Herrero-Ortega, A., Aleza, P., Pérez-Pérez, J., Ramón, D., Brunel, D., Luro, F., Chen, C., Farmerie, W. G., Desany, B., Kodira, C., Mohiuddin, M., Harkins, T., Fredrikson, K., Burns, P., Lomsadze, A., Borodovsky, M., Reforgiato, G., Freitas-Astúa, J., Quetier, F., Navarro. L., Roose, M., Wincker, P., Schmutz, J., Morgante, M., Machado, M. A., Talón, M., Jaillon, O., Ollitrault, P., Gmitter, F., & Rokhsar, D. (2014).** Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nature biotechnology*.

**Yamasaki, A., Kitajima, A., Ohara, N., Tanaka, M., & Hasegawa, K. (2007).** Histological study of expression of seedlessness in Citrus kinokuni 'Mukaku Kishu'and its progenies. *Journal of American Society for the Horticultural Science. 132*(6), 869-875.

**Yamasaki, A., Kitajima A., Ohara N., Tanaka M., Hasegawa K. (2009).** Characteristics of arrested seeds in Mukaku Kishu-type seedless citrus, *Journal of the Japanese Society for the Horticultural Science.*78 61–67.

**Yamaguchi, S. (2008).** Gibberellin metabolism and its regulation. *Annual Review of Plant Biology.*, *59*, 225-251.

**Yuan, L., & Xu, D. Q. (2001).** Stimulation effect of gibberellic acid short-term treatment on leaf photosynthesis related to the increase in Rubisco content in broad bean and soybean. *Photosynthesis Research*, *68*(1), 39-47.

**Zacarías, L., Talón, M., Ben-Cheikh, W., Lafuente, T., & Primo-Millo E. (1995).** Abscisic acid increases in non-growing and paclobutrazol-treated fruits of seedless mandarins. *Physiologia Plantarum* 95: 613–619.

**Zeevaart, J. A., & Gage, D. A. (1993).** Ent-Kaurene biosynthesis is enhanced by long photoperiods in the long-day plants Spinacia oleracea L. and Agrostemma githago L. *Plant physiology*, *101*(1), 25-29.

**Zhu, Y., Nomura, T., Xu, Y., Zhang, Y., Peng, Y., et al. (2006).** Elongated uppermost internode encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. *Plant Cell* 18: 442–456.